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13. ABSTRACT *(Maximum 200 Words)*

For many patients with mammary cancer the primary tumor can be successfully treated by surgical removal, however the long-term prognosis is not favorable because of the high frequency of metastatic disease, which is not treatable by current approaches. Our goal is to develop vaccination strategies to minimize metastatic disease. We postulate that an improvement in the generation of mammary carcinoma-specific $CD4^+$ T lymphocytes will facilitate development of $CD8^+$ T cell mediated immunity. We have generated vaccines consisting of mouse mammary tumor cells transfected with MHC class II, costimulatory (B7.1 or CD80), and superantigen (SEB) genes, and have shown that the vaccines directly present tumor encoded antigens to $CD4^+$ T cells.. To test vaccine efficacy, we have developed a mouse mammary carcinoma model that closely parallels human breast cancer. The BALB/c-derived 4T1 tumor spontaneously metastasizes throughout the body following inoculation into the mammary gland. Metastases are well established within 2-3 weeks of inoculation, and removal of primary tumor does not alter metastatic disease progression. Treatment with the vaccines significantly decreases the number of metastatic cells and increases survival time following surgical removal of primary tumor. Therapy with the class II/CD80 vaccine plus IL-12 also reduces metastatic disease, and probably involves chemokines in addition to T lymphocytes.

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FOREWORD

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INTRODUCTION

For many patients with mammary cancer the primary tumor can be successfully treated by surgical removal, however the long-term prognosis is not favorable because of the high frequency of metastatic disease which is not treatable by current approaches. We are using tumor-specific immunotherapy to curtail the incidence of metastatic breast cancer. Some of the most efficient anti-tumor mediators are tumor-specific $CD8^+$ T lymphocytes. In most cases, for optimal activity $CD8^+$ T cells require "help" from antigen-specific $CD4^+$ T lymphocytes (1-3). Recent studies indicate that the inability of the tumor-bearing host to reject tumors may be due to a lack of adequate tumor-specific T_h lymphocytes (3-6) We have therefore hypothesized that tumor-specific T_h activity can be significantly improved by generating tumor cells that contain all of the necessary antigen presentation, accessory and costimulatory molecules such that they are competent for tumor peptide presentation to $CD4^+$ T cells, and thereby facilitate T_h cell activation (reviewed in (7)). Such genetically engineered tumor cells could be used as vaccines to prevent development of metastatic breast cancer, and thereby enhance a host's tumor-specific immune response.

Our strategy is to genetically modify tumor cells so that they can directly present mammary carcinoma tumor peptides to $CD4^+$ T helper cells, thereby by-passing the requirement for professional antigen presenting cells and making more efficient the presentation of tumor peptides to T helper lymphocytes (reviewed in (7)). Accordingly, in the *first specific aim* we are using DNA-mediated gene transfer techniques to generate mammary tumor cell transfectants expressing many of the molecules constitutively expressed by professional antigen presenting cells (APC). These molecules include the peptide binding structures or MHC class II molecules, as well as the costimulatory molecule CD80 which has been shown to deliver the requisite second signal for T cell activation. In addition, the gene encoding the bacterial superantigen, SEB, a potent polyclonal T cell activator (8), is being transfected into the mammary tumor lines. The cytokine IL-12 (9, 10), a potent inducer of T_{h1} lymphocytes, will be used in conjunction with the tumor cell transfectants. In the *second specific aim* we are determining the tumorigenicity of the transfectants, and their ability to protect the syngeneic host against subsequent challenges of wild type tumor. We will also determine the ability of the transfectants to "rescue" mice carrying established wild type mammary tumors, and identify the helper and effector lymphocytes functional in mammary tumor rejection. In the *third specific aim* we are determining if metastatic mammary cancer can be reduced or prevented by immunization or concomitant treatment with the tumor cell transfectants. This novel tumor-specific immunotherapy approach should significantly improve the host's immune response to autologous breast tumor, and may provide several potential strategies for immune intervention in metastatic mammary cancer.

BODY

This is the final report and includes the four funded years plus an additional 1.5 years no-cost extension. This report contains a detailed description of the last 1.5 years of the project. The *Conclusion* section summarizes the project in its entirety.

During the past 1.5 years we have refined the 2^{nd} generation vaccines produced during the first years of this grant and have tested these vaccines extensively using the 4T1 mouse mammary tumor

model. Significant therapeutic effects were seen when the superantigen, SEB, from S. aureus was incorporated into the vaccines and when the vaccines were co-administered with the cytokine IL-12. As described in last year's report, following inoculation of the 4T1 tumor in the mammary gland of syngeneic BALB/c mice, tumor cells spontaneously metastasizes throughout the mouse. It was also shown in last year's report that mice with primary tumor develop metastases to the lungs within 2-3 weeks of implantation of 7 X 10^3 4T1 cells in the mammary gland (11). Surgical removal of the primary tumor does not alter the growth of metastatic cells, and mice typically die from metastatic disease within 42-48 days of initial tumor implantation in the mammary gland (12).

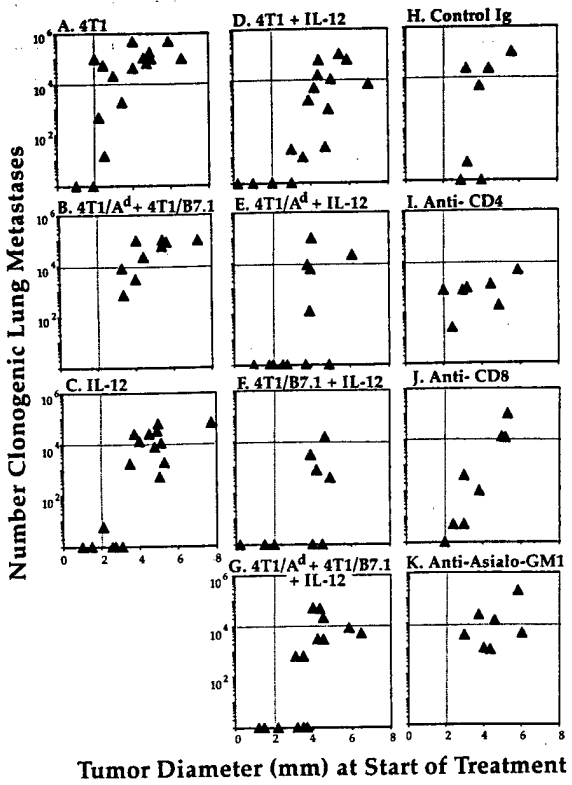

Figure 1. Therapy of established metastases with 4T1 plus IL-12 reduces the number of metastatic tumor cells in the lungs.

Therapy with 4T1 cells plus IL-12 reduces lung metastases in mice with spontaneous metastatic disease. Our cell-based vaccines were designed on the premise that activation of $CD4^+$ T cells would provide "help" to $CD8^+$ T cells and thereby facilitate tumor rejection. Because IL-12 is a potent cytokine for activating T_{h1} $CD4^+$ T cells, we reasoned that vaccine efficacy might be enhanced by co-administration of IL-12. To test this hypothesis, BALB/c mice were inoculated in the mammary gland with 7 X 10^3 4T1 cells and therapy with 4T1, 4T1/A^d, or 4T1/CD80 (4T1/B7.1) ± IL-12 started on day 21. Therapy consisted of 1 injection/week of cells plus 3 injections/week of 1 μg IL-12. After three weeks of therapy, the mice were sacrificed, their lungs removed, and the number of metastatic cells determined using the clonogenic assay (11). As shown in **figure 1** (panels A-G), treatment with any 4T1 cells (4T1/A^d, 4T1, or 4T1/CD80) plus IL-12 shows marked reduction in the number of clonogenic lung metastases relative to 4T1 only treated mice ($p < 0.05$). These studies are reported in (13).

The therapy effect of cells plus IL-12 is not exclusively depedent on $CD4^+$, $CD8^+$, or NK cells. To identify the cells responsible for decreased metastatic disease, mice undergoing therapy were depleted for $CD4^+$, $CD8^+$, or NK^+ cells by in vivo antibody treatment (14). As shown in **figure 1**(panels H-K), neither of these antibodies alone significantly

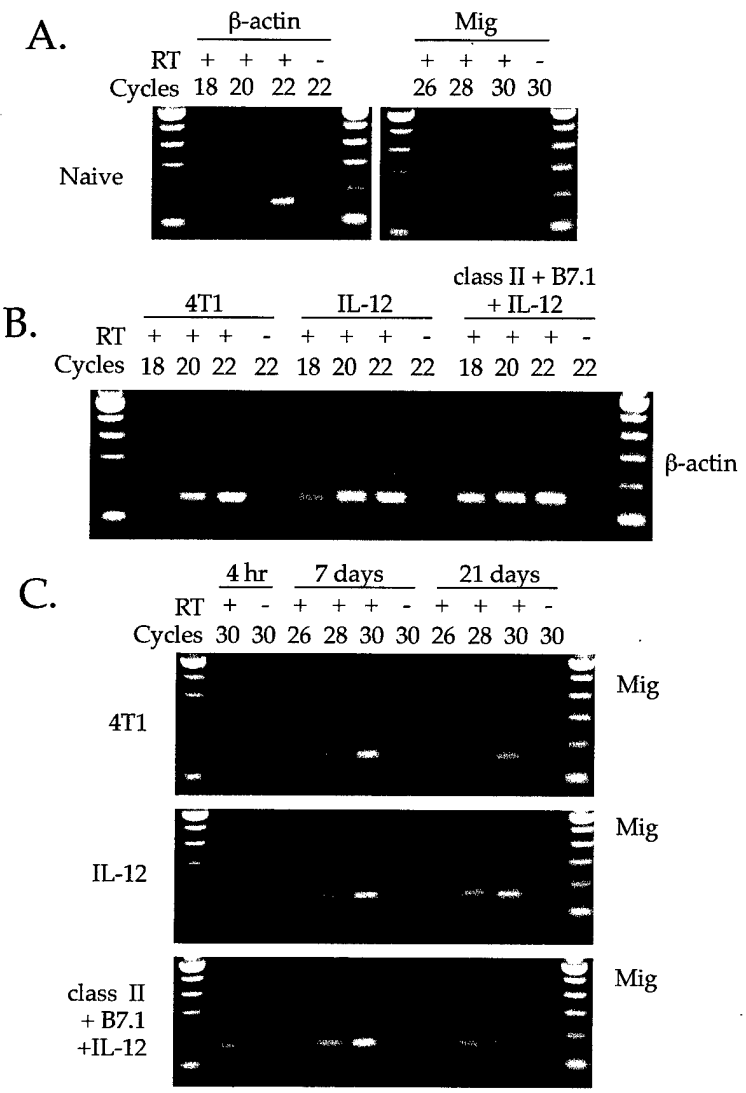

Figure 2. mRNA for Mig is expressed in the lungs of BALB/c mice with 4T1 metastases and receiving 4T1 plus IL-12 therapy. Panel A: Mig and β-actin in naive BALB/c mice. Panel B: β-actin in 4T1, IL-12-treated 4T1, and 4T1 plus IL-12 treated tumor-bearing mice. Panel C: Mig in therapy mice at 4 hours, 7 days, and 21 days after initiation of therapy.

samples at 4 hours, 7 days, and 21 days after initiation of therapy (**figure 2**, panel C), and was not present in the lungs of tumor-free mice (**figure 2**, panels A and B). Interestingly, 4T1 cells treated with IFN-γ express Mig (**figure 3**), suggesting that the tumor cells themselves may reduced the anti-tumor effect. As an additional measure of NK activity, BALB/c nude/beige/XID mice were also inoculated with 4T1 and therapy performed as described for figure 1. The therapy was equally effective in the nude/beige/XID mice as in BALB/c mice (data not shown). These results suggest that other factors and/or combinations of cells mediate metastasis reduction. These studies are reported in (13).

The chemokine Mig, produced by 4T1 cells, may be involved in the reduction of metastases via an anti-angiogenic mechanism. In addition to being a potent activator of T_{h1} cells, IL-12 induces interferon-γ (IFN-γ) which in turn stimulates the anti-angiogenic chemokines, monokine-induced by interferon (Mig) and IFN-γ-inducible protein-10 (IP-10) (15-17). We hypothesized that reduction in metastatic disease is the result of production of these anti-angiogenic factors. To determine if the 4T1 plus IL-12 therapy induces Mig and/or IP-10, RNA was isolated from the lungs of therapy mice and PCR amplified using Mig and IP-10 primers. To semi-quantify the amount of chemokine, PCR was performed for 26, 28, or 30 cycles. IP-10 was not expressed (data not shown), however, Mig was present in all

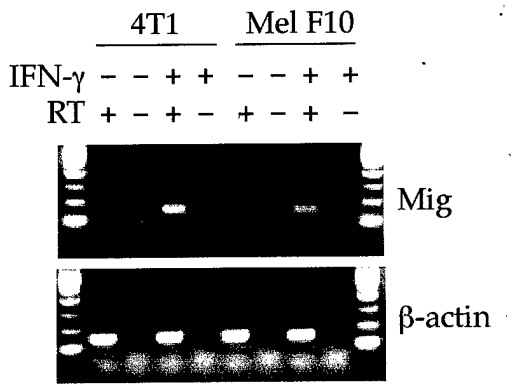

Figure 3. 4T1 tumor cells are induced in vitro by IFN-γ to express Mig. 4T1 cells were co-cultured with or without IFN-γ for 2 hours.

make the chemokine. These experiments suggest that the anti-tumor effect of IL-12 may be at least partially due to production of Mig in the lungs. These studies are reported in (13).

Post-operative therapy of mice with MHC class II$^+$CD80$^+$ transfectants secreting the superantigen, SEB, increases survival. *(Note: preliminary results of some of the following experiments were reported in the 1998 report.)* Superantigens, such as SEB, are polyclonal activators of T lymphocytes and have been shown to activate tumor-specific T cells (8). We have hypothesized that 4T1 tumor cells secreting SEB may be effective vaccines for activating tumor-specific T lymphocytes. MHC class II and CD80 expression of the 4T1/SEB transfectants was shown in the 1998 report. To determine if the SEB transfectants secrete SEB, supernatants of 4T1/SEB cultures were tested for their ability to stimulate naive T cell proliferation. As shown in **Figure 4**, supernatants of two 4T1/SEB lines (#12 and 14) stimulate T cell proliferation as measured by MTT assay (18). The proliferation is blockable by antibodies to SEB, demonstrating that the proliferation is due to SEB production by the transfectants. The therapeutic efficacy of the SEB transfectants was tested in mice with established metastatic disease. BALB/c mice were inoculated in the mammary gland with wild type 4T1 tumor and primary tumors allowed to progress for 3 weeks at which time they were surgically removed. One week later therapy with 4T1/A^d/B7.1 plus 4T1/SEB cells was started. As shown in **Figure 5**, therapy with 4T1/A^d/B7.1 plus 4T1/SEB cells (panel D) significantly extends survival time to 41-74 days from 35-52 days for 4T1 treated mice (p,0.05). These studies are reported in (12).

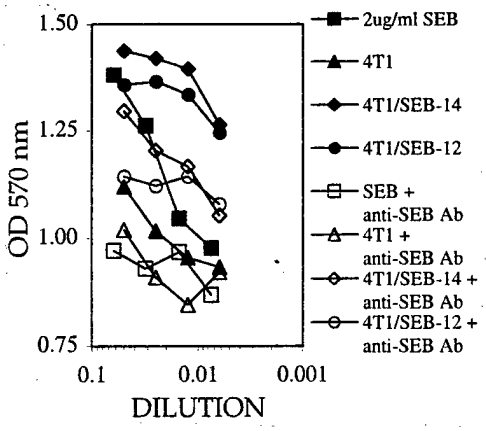

Figure 4. 4T1/SEB cells secrete SEB that stimulates T cell proliferation. Purified SEB and supernatants of 4T1/SEB cells were co-cultured with naive splenocytes. Antibodies to SEB were added to some cultures to demonstrate specificity of T cell proliferation.

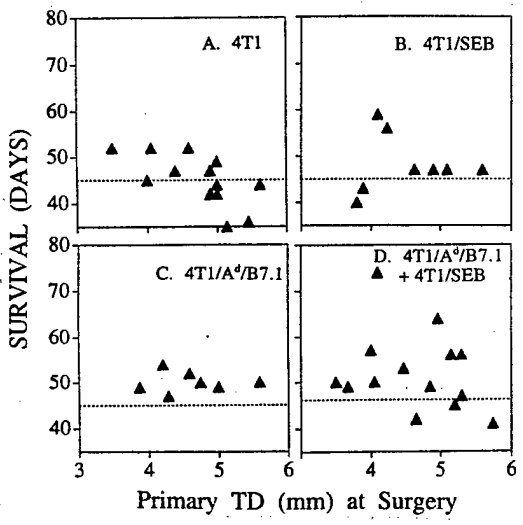

Figure 5. Immunotherapy of established, disseminated mammary carcinoma with 4T1/A^d/B7.1 plus 4T1/SEB increases survival time.

Increase in survival time is due to a decrease in the number of distant metastases and is mediated by CD4$^+$ and CD8$^+$ T cells. To ascertain that the increase in survival time is the result of reduced metastatic disease, the number of clonogenic metastatic cells in the lungs of therapy mice was measured. BALB/c mice were inoculated in the mammary gland with wild type 4T1 tumor, and therapy with 4T1/A^d/B7.1 plus 4T1/SEB started on day 14, at which time distant metastases are well established (11). Therapy was administered twice a week until day 42, at which time mice were sacrificed, their lungs removed, and the number of clonogenic metastatic cells in the lungs determined using the 6-thioguanine assay (11). As shown in **Figure 6**, mice treated with the combination therapy (4T1/A^d/B7.1 plus 4T1/SEB; panel D) have significantly fewer metastatic cells in their lungs than mice treated with either transfectant alone or with wild type 4T1

(panels A-C) ($p < 0.05$).

We have hypothesized that SEB improves anti-tumor immunity because it enhances activation of $CD4^+$ T cells specifically activated by the 4T1/A^d/B7.1 vaccine. To ascertain that $CD4^+$ T cells are involved in the anti-tumor effect, therapy experiments were conducted in mice depleted for $CD4^+$ or $CD8^+$ T cells. BALB/c mice were inoculated in the mammary gland with wild type 4T1 cells and cell therapy started on day 14 and continued throughout the experiment. Mice were depleted for $CD4^+$ or $CD8^+$ T cells by administration of antibody depletion as previously described (14). Mice were sacrificed on day 42, their lungs removed and the number of metastatic cells in the lungs determined by the 6-thioguanine assay (11). As shown in **Figure 7**, mice treated with the combination therapy have significantly reduced numbers of metastatic cells (panel B) relative to mice treated with wild type 4T1 cells (panel A). In contrast, mice depleted for $CD4^+$ or $CD8^+$ T cells and nude mice have numbers of metastatic cells in their lungs comparable to 4T1 treated mice (panels C, D, and F, respectively). These experiments demonstrate that increased survival is due to decreased metastatic disease and that $CD4^+$ and $CD8^+$ T cells are critical. These studies are reported in (12).

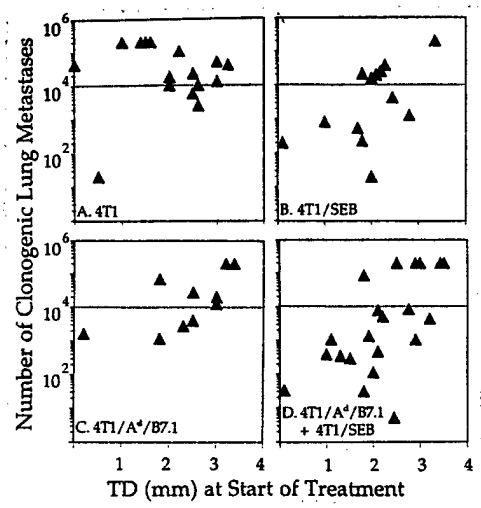

Figure 6. Immuntherapy of BALB/c mice with disseminated, advanced metastatic disease with 4T1/A^d/B7.1 plus 4T1/SEB reduces the number of metastatic cells in the lungs.

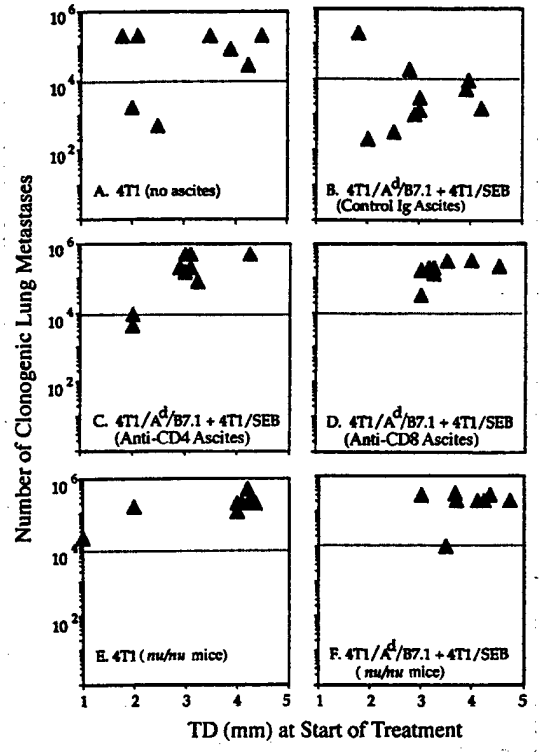

Figure 7. Reduction in metastatic disease following therapy with 4T1/A^d/B7.1 plus 4T1/SEB vaccines requires $CD4^+$ and $CD8^+$ T cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Establishment of the mouse 4T1 mammary carcinoma as a post-surgical model for spontaneous mammary metastases
- Development of MHC class II/CD80 cell-based vaccines as immunotherapy agents for the treatment of metastatic breast cancer
- Combination therapeutic agent of MHC class II vaccines plus the superantigen SEB

REPORTABLE OUTCOMES:

- See lists of refereed manuscripts and conference presentations under *#13 Final Reports* section.

See attached manuscripts in the Appendix for detailed descriptions of the following scientific findings:

- Development of the mouse 4T1 mammary carcinoma as a post-surgical animal model.
- Development of cell based vaccines consisting of MHC class II, CD80, and SEB transfected tumor cells.
- Testing of the vaccines in syngeneic mice with extensive, disseminated mammary carcinoma metstases.
- Studies completed under this grant provide the framework for the following grant submissions:
- DOD USAMRMC Breast Cancer IDEA application, June 2000
- NIH R01 application, July, 2000
- DOD USAMRMC Breast Cancer Undergraduate Research Training application, June 2000.

CONCLUSIONS

During the final year of this grant we have completed most of the studies proposed in the original application. During the course of the grant period we have developed a mouse model for metastatic mammary carcinoma that closely parallels human metastatic breast cancer. The 4T1 tumor was originally isolated by Dr. Fred Miller from a spontaneous mammary carcinoma in BALB/c mice. It forms a primary, solid mass when inoculated into the mammary gland and spontaneously metastasizes throughout the host (lungs, liver, brain, lymph nodes, blood, etc.). Because tumor cells are resistant to 6-thioguanine, it is feasible to precisely quantify the number of metastatic cells in distant organs before they form visible masses (11). We have shown that the course of metastasis is not affected by surgical removal of the primary tumor, and that mice die from metastatic disease even when the primary tumor is removed (12). We have used this tumor model at a very advanced stage of disseminated metastatic disease to test several cell-based vaccines. *The extent of metastatic disease in the animals at the initiation of therapy is comparable to stage IV human breast cancer, and is significantly more advanced than metastatic disease in other published animal models.* The vaccines are based on the hypothesis that activation of tumor-specific $CD4^+$ T cells facilitates anti-tumor immunity. The basic vaccine consists of MHC class II and CD80 transfected 4T1 cells. We hypothesized that these cells directly present tumor associated peptides to $CD4^+$ T cells along with a required costimulatory signal and thereby specifically activate the $CD4^+$ T cells. This hypothesis has been confirmed in studies using a mouse sarcoma tumor (19-22). The basic vaccine has been supplemented with superantigen (SEB) transfected 4T1 tumor cells or with IL-12. Addition of IL-12 or SEB greatly enhances therapeutic efficacy and leads to significant extension of survival time (12, 13). Because we see significant tumor reduction in this very advanced animal model, we are encouraged that this vaccine strategy may have therapeutic efficacy in patients, and hence we will extend our studies to patients with metastatic breast cancer.

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APPENDICES

Copies of the publications listed in *Bibliography of All Publications, Refereed Papers* are appended. (Note: These publications contain their own pagination and are not paginated within the context of this Final Report.)

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Rejection of MHC Class II-Transfected Tumor Cells Requires Induction of Tumor-Encoded B7–1 and/or B7–2 Costimulatory Molecules[1]

Sivasubramanian Baskar,* Virginia K. Clements,* Laurie H. Glimcher,† Nasrin Nabavi,‡ and Suzanne Ostrand-Rosenberg[2]*

Many tumor cells that have been transfected with genes encoding B7 costimulatory molecules become effective cellular vaccines against wild-type tumor. The improved immunity is dependent on newly induced tumor-specific $CD8^+$ and/or $CD4^+$ T cells and presumably occurs because the B7 transfectants provide the requisite second signal for activation of T cells in conjunction with tumor cell-presented MHC class I/tumor peptide and/or MHC class II/tumor peptide complexes, respectively. Since B7 expression is such a potent enhancer of tumor immunity, and yet some tumors are immunogenic in the absence of B7 transfection, we have used class I^+class II-transfected tumors to investigate whether costimulatory molecules are also involved in rejection of immunogenic, non-B7-transfected tumor. Blocking studies with B7 mAbs demonstrate that induction of tumor immunity in naive mice requires B7–1 and/or B7–2 expression, while experiments with tumor-primed mice indicate that once antitumor immunity is established, expression of B7 is not necessary. Flow cytometry analyses demonstrate that costimulatory molecules are expressed by the tumor cells via an in vivo induction process. Experiments with class II genes with truncated cytoplasmic tails indicate that the cytoplasmic region of the tumor-expressed class II heterodimer is involved in induction of B7. We therefore conclude that for this class I^+class II-transfected tumor, generation of tumor immunity requires induction of tumor cell-encoded B7 molecules that are mediated by the cytoplasmic region of the transfected class II heterodimer. *The Journal of Immunology,* **1996, 156: 3821–3827.**

Tumor cell expression of B7 family members (B7-1 or CD80, B7-2 or CD86) via gene transfection or transduction has resulted in immune-mediated rejection of a variety of experimental tumors via activation of tumor-specific $CD4^+$ and/or $CD8^+$ T lymphocytes. Class I^+ tumor cells transfected with B7-1 usually exclusively activate $CD8^+$ T cells, while B7-1-transfected class I^+class II^+ tumors activate both $CD4^+$ and $CD8^+$ T cells (1–5). Such genetically modified tumor cells are themselves rejected by the autologous host, and immunization with them results in potent, long-lasting, tumor-specific immunity against the wild-type, unmodified parental tumor (1–4). Although expression of B7 family member genes has not succeeded in rendering all tested tumors immunogenic (5), this approach has yielded sufficient therapeutic results in experimental systems that it is considered a potentially promising strategy for tumor-specific immunotherapy.

Since antigen-specific T lymphocyte activation minimally requires an Ag-specific signal and a costimulatory signal, it is likely that the B7 tumor transfectants/transductants activate the host's immune system because they facilitate delivery of the costimulatory signal (1–3, 6). Presumably, $B7^+$class I^+ tumors provide a costimulatory signal that complements the Ag-specific signal delivered via the class I/tumor peptide complex, thereby activating $CD8^+$ tumor-specific T cells. $B7^+$class I^+class II-transfected tumors presumably costimulate via both class I/tumor peptide and class II/tumor peptide complexes and thereby activate both $CD4^+$ and $CD8^+$ tumor-specific T cells. Although this hypothesis suggests that B7 molecules play a critical role in mobilizing the immune response against autologous tumor, it also raises two fundamental questions about the function of B7 in tumor rejection. 1) Is expression of B7 molecules simply a convenient method for inducing tumor-specific immunity, or are B7 molecules also critically involved in immune-mediated rejection of constitutively immunogenic tumors? 2) Are there other molecules besides B7 family members that can provide the required costimulatory signal to activate tumor-specific immunity? In this study we address these closely related questions and report that induction of B7 molecules occurs during rejection of immunogenic tumors, and that for at least the SaI tumor, expression of B7 is necessary and sufficient for tumor rejection. These results further support the hypothesis that B7 family members are critical molecules for activating tumor-specific immunity.

Materials and Methods

Mice

Mice were obtained and maintained as previously described (6). Male and female mice, 6 to 16 wk of age, were used.

Tumors

Syngeneic A/J mice were inoculated i.p. with 1 to 2.5 × 10^6 SaI or SaI-derived tumor cells as previously described (3) and monitored for tumor

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growth 3 times per week. Mice that became moribund due to tumor (usually between 18 and 28 days after tumor challenge) were considered tumor susceptible and were killed according to IACUC guidelines. The remaining mice were followed for a minimum of 90 days post-tumor challenge and were considered tumor resistant if they did not develop tumor during this period.

Cells and transfections

The murine sarcoma tumor cell lines SaI, SaI/A^k (clone 19.6.4), SaI/A^ktr (clone 6.11.8), and SaI/A^ktr/B7-1 (clone 1) were maintained in vitro as previously described (3, 7, 8). SaI/B7-2 and SaI/A^k/B7-2 cell lines were generated by transfecting SaI and SaI/A^k tumor cells, respectively, with the pCEXV-3 plasmid containing the B7-2 cDNA (9). Transfectants expressing one wild-type class II chain and one truncated chain were prepared using cDNAs, as previously described (10). All transfectants were prepared by cotransfection with the pSV2hph plasmid (B7-2 transfectants) or the pSV2neo plasmid (truncates) and selected for growth in medium supplemented with 400 μg/ml hygromycin and/or G418 as previously described (3, 7). Tumor cell inoculations were performed as previously described (7).

Antibodies

The following mAb were used: B7-1 (1G10; 11), B7-2 (2D10; 12), MHC class II A^k (10-3-6; 13), CD3 (500A2; 14), CD4 (GK1.5; 15), CD8 (2.43; 16), B220 (17), and Mac-1 (18). In vivo Ab treatments with 1G10 and 2D10 were performed as previously described for CD4 and CD8 T cell depletions (3), with the following modifications. Mice received 100 μg of purified Ab (1G10, 2D10, or both) 2 to 3 days before and on the day of tumor challenge and twice a week thereafter until completion of the experiment. Control mice received 100 μl of PBS instead of Ab.

Immunofluorescence

Indirect immunofluorescence was performed as previously described using appropriate second Ab such as goat-anti-mouse IgG-FITC, mouse-anti-rat IgG-FITC, or goat anti-hamster Ig-FITC (7). Labeled cells were analyzed using a Coulter Epics XL or Epics C flow cytometer (Hialeah, FL). The percentage of positive cells was calculated using the Coulter Immuno-4 program or by gating against samples labeled with conjugate alone or irrelevant control isotype-matched Abs.

Analysis of B7 on in vivo-passaged tumor cells

Mice were inoculated i.p. with 1 to 2.5 $\times$ 10^6 tumor cells, and peritoneal exudate cells (PEC)[3] were removed 20 to 96 h later and tested by indirect immunofluorescence for B7-1 and B7-2. PEC were enriched for tumor cells in two different ways. In some experiments, PEC were plated 1 to 2 times in plastic tissue culture dishes for 30 to 60 min at room temperature or at 37°C to remove adherent cells. In other experiments, PEC were layered on iodixanol discontinuous density gradients (Accurate Chemical and Scientific Co., Westbury, NY), and the cells migrating at 1.0575 g/ml were harvested. This population consisted almost entirely of SaI-derived tumor cells. Tumor cells in the peritoneal exudate were identified by flow cytometry, based on their significantly larger size as compared with host cells in the peritoneal exudate, and bit-mapped accordingly. Nontumor cells in the peritoneal exudates were identified by forward and side scatter bit-mapping and fluorescence labeling with mAb to CD3, CD4, CD8, Mac-1, and B220. Comparisons were also run between PEC from tumor-inoculated vs non-tumor-inoculated mice to ascertain the side scatter and forward light scatter parameters of tumor cells. The final population of cells in the "tumor cell" bit map was greater than 95% tumor cells as assessed by size, side scatter, and lack of staining for markers of other cell types.

For the transfectants expressing truncated α- or β-chain genes, multiple clones were tested (see Table IV). Since, on repeated experiments, independent clones of a given genotype gave similar results, B7-1 and B7-2 induction measurements for separate clones were pooled.

Results

Tumor rejection by naive mice requires expression of B7-1 and/or B7-2, while rejection by tumor-primed mice is independent of B7-1/B7-2 expression

In our previous studies, we have demonstrated that expression of syngeneic MHC class II genes via tumor cell transfection results in genetically modified tumor cells that are potent tumor vaccines (7).

[3] Abbreviation used in this paper: PEC, peritoneal exudate cell.

Table I. *In vivo treatment was Abs to B7-1 plus B7-2 prevents tumor rejection in naive mice, but not in mice primed to tumor*[a]

Mice	Tumor Challenge	mAb Treatment	Tumor Incidence
Naive	SaI/A^k	Untreated	1/10
		Anti-B7-1	0/10
		Anti-B7-2	0/5
		Anti-B7-1 + anti-B7-2	12/12
Naive	SaI/A^ktr/B7-1	Untreated	0/5
		Anti B7-1	4/5
Tumor-primed	SaI/A^k	Untreated	0/5
		Anti-B7-1 + anti-B7-2	0/5

[a] Naive A/J mice were treated with Abs to B7-1 (1G10) and/or B7-2 (2D10), challenged with 10^6 SaI/A^k or SaI/A^ktr/B7-1 tumor cells, and followed for tumor incidence. Tumor-primed mice were immunized with 10^6 SaI/A^k tumor cells 3 wk prior to initial Ab treatment.

Additional studies with sarcoma tumor cells expressing class II molecules with truncated cytoplasmic domains implicated B7 as a critical molecule in the induction of immunity (3). Because B7 molecules on B7 transfectants appear to be involved in the induction of tumor-specific immunity, we have investigated whether they are required for the rejection of immunogenic (non-B7-transfected) tumor cells.

Autologous naive A/J mice were either untreated, given mAb to B7-1 (1G10 mAb) or B7-2 (2D10 mAb), or given a combination of 1G10 plus 2D10 and challenged i.p. with 10^6 live SaI/A^k tumor cells. If expression of B7-1 and/or B7-2 is necessary for tumor rejection, then blocking expression with Ab may result in tumor growth. As shown in Table I, untreated mice or mice given either of the Abs alone reject the SaI/A^k tumor, while mice given the two mAbs in combination develop tumors. Rejection of the class II^+ SaI/A^k sarcoma by naive autologous mice therefore requires expression of either B7-1 or B7-2 molecules, and additional costimulatory signals are not needed.

In previous studies, we have shown that SaI/A^k cells, in which the class II molecules have truncated cytoplasmic regions (SaI/A^ktr cells), are highly tumorigenic (8). However, if transfected with B7-1, the resulting tumor cells are rejected (SaI/A^ktr/B7-1; Ref. 3). These experiments were interpreted to indicate that co-expression of B7-1 restored the immunogenic phenotype to the tumor cells. To test whether B7-1 is essential for immune rejection of the SaI/A^ktr/B7-1 tumor cells, we also treated naive mice with mAb to B7-1 and subsequently challenged with SaI/A^ktr/B7-1 tumor. As shown in Table I, mAb treatment blocks tumor rejection, thereby demonstrating a critical role for B7-1 expression by SaI/A^ktr/B7-1 cells.

To determine the role of B7 molecules in tumor rejection by tumor-immunized mice, autologous A/J mice were first primed to the SaI/A^k tumor, subsequently given B7-1 and B7-2 mAbs, and then challenged with SaI/A^k tumor. As shown in Table I, immunized mice treated with mAb to B7-1 and B7-2 remain tumor-free, indicating that B7 molecules are not required for rejection of a secondary tumor challenge by tumor-primed mice. Expression of either B7-1 or B7-2 molecules is therefore critical for rejection of tumor by naive mice but is not essential for tumor rejection by tumor-primed mice.

B7-2-transfected SaI sarcoma cells are as immunogenic as B7-1-transfected tumor cells

The B7 blocking experiments shown in Table I indicate the involvement of B7-2 as well as B7-1 in the development of tumor immunity. To directly test whether expression of B7-2 is as potent

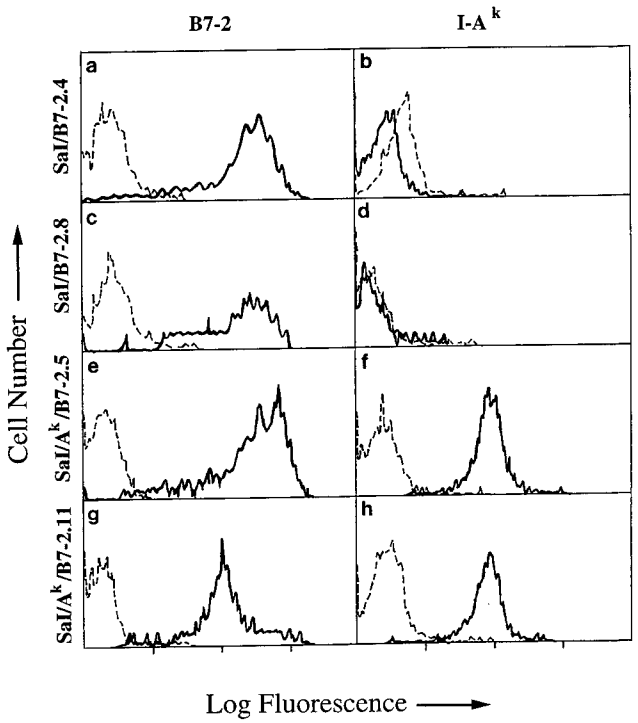

FIGURE 1. Sal and Sal/A^k tumor cells transfected with MHC class II and/or B7-2 genes (Sal/B7-2 and Sal/A^k/B7-2 lines) express class II and B7-2 molecules. Sal/B7-2 (clones 4 and 8, *a–d*) and Sal/A^k/B7-2 (clones 5 and 11, *e–h*) transfectants were labeled with 2D10 mAb to B7-2 (*a, c, e,* and *g*) or 10-3-6 mAb to I-A^k (*b, d, f,* and *h*). Dotted lines represent staining by fluorescent conjugate alone; solid lines represent staining with conjugate plus mAb.

an inducer of tumor immunity as is B7-1, B7-2-transfected Sal and Sal/A^k tumor cells were generated and their tumorigenicity tested. Figure 1 shows the flow cytometry profiles of two Sal/B7-2 (clones 4 and 8) and two Sal/A^k/B7-2 (clones 5 and 11) transfectant clones labeled for B7-2 (Fig. 1, *a, c, e,* and *g*) and MHC class II (I-A^k; Fig. 1, *b, d, f,* and *h*) molecules. To test the tumorigenicity of these clones, naive A/J mice were challenged i.p. with 10^6 transfectants, then followed for tumor development. As shown in Table II, all of the B7-2 transfectants were rejected. To test the immunogenicity of the transfectants, naive A/J mice were immunized i.p. with 10^6 transfectants, then challenged 5 to 8 wk later with 10^6 wild-type live Sal tumor. As shown in Table II, mice immunized with either of the transfectants are immune to subsequent challenge with wild-type sarcoma, while unimmunized A/J mice are highly susceptible. B7-2 transfectants also cause tumors in nude mice (data not shown), indicating that rejection of B7-2 transfectants by A/J mice is T cell mediated. Sal/B7-2 and Sal/A^k/B7-2 transfectants are, therefore, also potent vaccines against wild-type Sal sarcoma and appear to be as effective immunogens as B7-1 transfectants.

During the rejection process, class II^+ tumor cells are induced to express B7-1 and B7-2 molecules

Experiments with B7-1- and B7-2-transfected tumor cells (1–4, 29; Table II) suggest that tumor cell expression of B7-1 and/or B7-2 via gene transfection is extremely advantageous for the induction of tumor immunity. Although B7 transfection is a convenient method for enhancing antitumor immunity, it is not clear whether B7 expression accompanies normal tumor rejection, i.e., rejection in the absence of transfected B7. The mAb-blocking experiments shown in Table I support the hypothesis that B7-1 and/or B7-2 expression is critical for tumor rejection; however, these experiments do not identify the cells that express B7. Since Sal and its derivatives grow as ascites tumors in the peritoneal cavity, we have reasoned that the cells (either host or tumor) that express B7 molecules will be present in the peritoneal exudate following inoculation of the tumor cells. To identify the cells that express B7, we therefore inoculated mice i.p. with Sal/A^k tumor and removed PEC at varying time points after inoculation, monitoring them by indirect immunofluorescence for B7-1 and B7-2 expression. Since the peritoneal exudate includes a mixture of various cell types, we used a variety of methods to distinguish tumor cells from host cells. These methods include flow cytometry bit-mapping by size and side scatter, immunofluorescence staining for T cell Ags ($CD4^+$, $CD8^+$, CD3), macrophage Ag (MAC-1), and B cell Ag (B220), and physical separation of cells by plastic adherence or density gradient centrifugation. Because Sal/A^k tumor cells are significantly larger and less dense than host PEC, they are readily identifiable by flow cytometry and can be separated by density gradient centrifugation.

Table II. *Immunization with B7-2-transfected sarcoma cells protects against challenge of wild-type tumor*[a]

Immunizing Tumor	Challenge Tumor	Tumor Incidence
	Sal	10/10
	Sal/B7-2.4	0/15
	Sal/B7-2.8	0/10
	Sal/A^k/B7-2.5	0/15
	Sal/A^k/B7-2.1	0/15
None	Sal	10/10
Sal/B7-2.4	Sal	0/15
Sal/B7-2.8	Sal	0/15
Sal/A^k/B7-2.5	Sal	1/15
Sal/A^k/B7-2.11	Sal	1/10

[a] Naive A/J mice were challenged i.p. with 10^6 transfectants. Immunized A/J mice were inoculated i.p. with 10^6 transfectants and challenged i.p. 35 days later with 10^6 wild-type live Sal tumor cells. Unimmunized control mice were challenged with 10^5 or 10^6 Sal cells. Mice not developing tumor remained tumor-free for longer than 6 mo.

A/J mice were inoculated i.p. with 1 to 2.5 × 10^6 tumor cells, and peritoneal exudates were removed 24 to 96 h later. PEC were plated on plastic to remove adherent cells and subsequently tested by indirect immunofluorescence for B7-1 and B7-2 expression. In preliminary experiments, it was noted that the PEC from mice inoculated with either Sal or Sal/A^k cells contained $B7^+$ cells in the macrophage population, but only Sal/A^k-inoculated mice contained $B7^+$ cells in the tumor population (data not shown). Macrophage-encoded B7 therefore appeared similar in resistant and susceptible mice, while tumor cell-encoded B7 differed. Subsequent experiments therefore focused on the tumor cell subset of the exudate.

As shown in Figure 2, *c* and *d*, a significant subset of immunogenic Sal/A^k tumor cells passed in vivo for 48 h become B7-1- and B7-2-positive, while Sal cells do not (Fig. 2, *g* and *h*). Sal/A^k tumor cells passed in vivo for 72 h are also B7-1- and B7-2-positive; however, by 96 h, very few Sal/A^k tumor cells could be recovered from the peritoneal cavity, and hence B7-1 and B7-2 expression could not be measured (data not shown). Neither tumor population expresses B7-1 or B7-2 when passed in culture (Fig. 2, *a, b, e,* and *f*). Because of the proposed involvement of the cytoplasmic domain of the class II heterodimer in the induced expression of B7 molecules (11), we also tested Sal cells transfected with class II genes that have truncated cytoplasmic domains (Sal/A^ktr cells; 8). As shown in Figure 2, *k* and *l*, Sal/A^ktr cells grown in

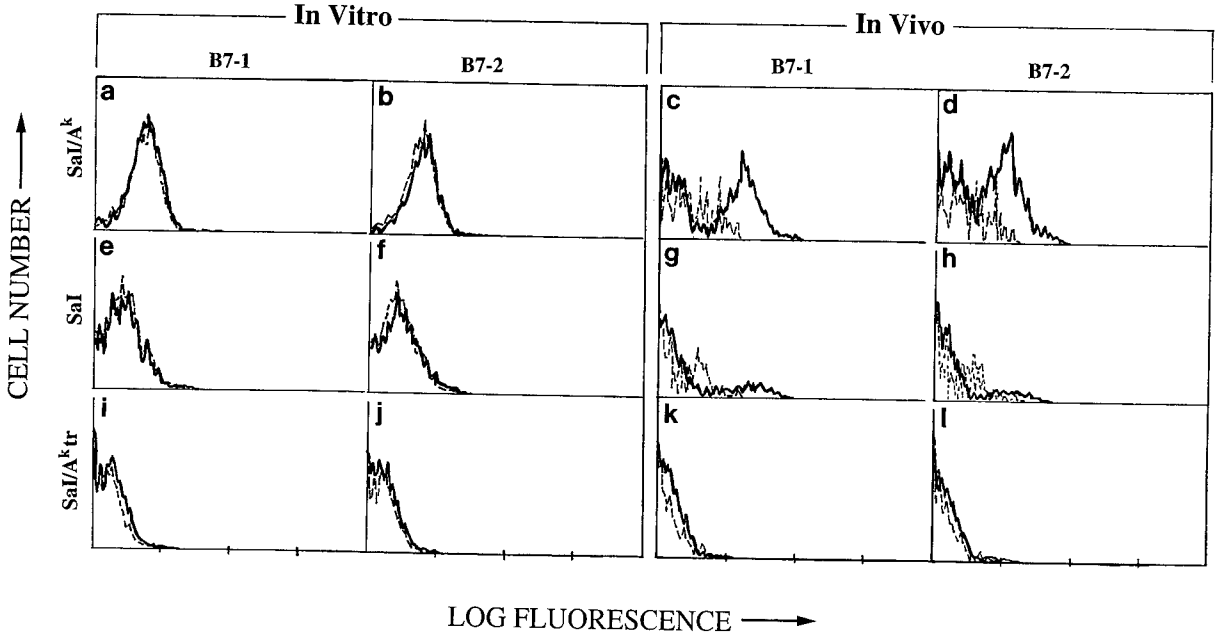

FIGURE 2. B7-1 and B7-2 molecules are induced on SaI/A^k tumor cells during tumor rejection. A/J mice were inoculated i.p. with 1 to 2.5 × 10^6 SaI/A^k (*c* and *d*), SaI (*g* and *h*), or SaI/A^{k}tr (*k* and *l*) cells on day 0, and PEC were removed 40 to 48 h later. Tumor cells were enriched in the PEC population as described in *Materials and Methods* and the resulting cells labeled with mAbs to B7-1 (*c, g,* and *k*) or B7-2 (*d, h,* and *l*). For comparison, in vitro-cultured SaI/A^k (*a* and *b*), SaI (*e* and *f*), and SaI/A^{k}tr (*i* and *j*) were also stained for B7-1 or B7-2. Dotted lines represent staining by conjugate alone; solid lines represent staining with mAb plus conjugate.

Table III. *B7-1 and B7-2 molecules are induced on SaI/A^k tumor cells in vivo*[a]

Tumor Cells	Genotype	Time In Vivo (h)	Percent Positive Cells ±SE B7-1	B7-2
SaI/A^k	Wild-type	20–24	7.3 ± 2.2	18 ± 5
		40–48	13.5 ± 3.6	25.1 ± 5.6
SaI/A^{k}tr	Truncated α- and β-chains	20–24	1.8 ± 0.5	6.7 ± 3.6
		40–48	2.6 ± 2.6	1 ± 1
SaI	Class II	20–24	4 ± 1	4 ± 0.6
		40–48	2 ± 1	1.5 ± 0.5

[a] A/J mice were inoculated i.p. with 2 × 10^6 tumor cells. Peritoneal exudates were removed 1 to 2 days later, and cells were labeled for immunofluorescence. Tumor cells were distinguished from other cells in the perioteneal exudate, and percent cells staining for B7-1 and B7-2 was calculated as described in *Materials and Methods*.

Table IV. *Genotypes of sarcoma (SaI) transfectants used in these studies*

Cells	Clones	Genotype of Transfected MHC Class II Genes
SaI/A^k	19.6.4	Wil-type α- and β-chains
SaI/A^{k}tr	6.11.8	α- and β-chains truncated for 12 and 10 aa, respectively, at cytoplasmic end
SaI/A^{k}βtr	12-10	Wild-type α-chain; β-chain truncated for 10 aa at the cytoplasmic end
	12-11	
SaI/A^{k}αtr	8.7	α-chain truncated for 12 aa at the cytoplasmic end; wild-type β chain
	8.12	
	8.2	
	8.26	

aa, amino acids.

vivo for 48 h do not express B7 molecules and remain as negative for B7-1 and B7-2 as the in vitro-cultured SaI/A^{k}tr tumor cells (*i* and *j*).

Each flow cytometry profile in Figure 2 represents tumor cells passed in an individual mouse. To obtain a more statistically meaningful result, induction data from a minimum of 10 mice (range, 10–30) per cell type were pooled. These results are presented in Table III. Tumor cells were inoculated i.p. (1–2.5 × 10^6 cells) and removed 20 to 24 or 40 to 48 h later for immunofluorescence staining for B7-1 and B7-2 molecules. Numbers are the percentage of B7 positive cells in the total tumor cell component of the peritoneal exudate. Although there is variation between individual mice, these results are consistent with the flow cytometry profiles shown in Figure 2. During the rejection process, a subpopulation of immunogenic class II$^+$ SaI/A^k cells therefore becomes B7-1- and B7-2-positive, while nonimmunogenic SaI and SaI/A^{k}tr cells remain negative for B7 expression.

The cytoplasmic domain of the class II molecule of SaI sarcoma cells is involved in induction of B7-1 and B7-2

Wild-type MHC class II molecules consist of an α- and a β-chain that span the plasma membrane and extend for approximately 18 amino acids into the cytoplasmic compartment. A previous study (11) suggested that induction of B7 expression is mediated by signals transmitted via the cytoplasmic domain (carboxyl terminus) of the MHC class II heterodimer. The SaI/A^{k}tr data of Table III confirm that the class II cytoplasmic region is involved in induction of B7 on sarcoma cells.

To further localize the class II region required for B7 induction, SaI tumor cells expressing MHC class II heterodimers, consisting of one wild-type chain and one chain truncated at its carboxy (cytoplasmic) terminus, were generated by gene transfection (SaI/A^{k}αtr, SaI/A^{k}βtr). Table IV summarizes these cell lines and their class II genotypes, and Figure 3 shows flow cytometry profiles of the transfectants for MHC class II Ag expression. Since the SaI/A^{k}αtr and SaI/A^{k}βtr clones show very similar MHC class II staining with the 10-3-6 mAb, only one clone of each cell type is

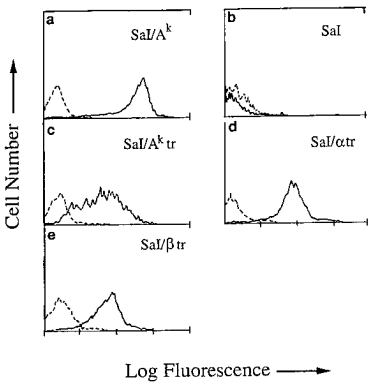

FIGURE 3. Immunofluorescence staining of SaI and class II-transfected SaI cells for I-A^k expression. Cells were stained with the 10-3-6 mAb to I-A^k. Since the two SaI/$A^k\beta$tr clones (12-10 and 12-11) had very similar staining profiles for I-A^k, and all four of the SaI/$A^k\alpha$tr clones (8.7, 8.12, 8.2, and 8.26) had similar staining for I- A^k, only one clone of each transfectant is shown. Dotted lines represent staining by fluorescent conjugate alone; solid lines represent staining by mAb plus conjugate.

Table V. *B7-1 and B7-2 molecules are induced on sarcoma cells with truncated or mutant α- or β-chains*[a]

Tumor Cells	Class II Genotype	Time In Vivo (h)	Percent Positive Cells ±SE B7-1	B7-2
SaI/$A^k\beta$tr	α-wild-type	20-24	6.2	4.8
	β-truncated	40-48	12.0 ± 4.9	8.6 ± 1.1
SaI/$A^k\alpha$tr	α-truncated	20-24	1 ± 0	4.2 ± 0.6
	β-wild-type	40-48	5.9 ± 1.1	4.5 ± 1.2

[a] A/J mice were inoculated i.p. with 2×10^6 tumor cells. Peritoneal exudates were removed 1 to 2 days later and remaining cells label for immunofluorescence. Tumor cells were distinguished from other cells in the peritoneal exudate, and percent cell staining was calculated as describe in *Materials and Methods*. Data on several clones of each tumor type were pooled.

shown. If the truncations eliminate regions of the class II molecule that are critical for induction of B7 expression, then SaI cells expressing these genetically modified molecules should not induce B7 expression. A/J mice were therefore inoculated i.p. with 2×10^6 tumor cells, and the tumor cells were removed 1 to 2 days later and tested by indirect immunofluorescence for B7-1 and B7-2 expression. All of the clones listed in Table IV, except SaI/$A^k\beta$tr 12-11, were tested. Since the results for the clones of each genotype were similar (including the SaI/$A^k\alpha$tr 8.2 clone), induction values for individual clones of each genotype were pooled and are shown in Table V. SaI/A^ktr tumor cells are induced for B7 molecules; however, the level of induction is much lower than that seen for tumor cells with full-length α- and β-chains (see Table III for SaI/A^k values). The B7 induction level for SaI/$A^k\alpha$tr cells is even lower; however, at 40 to 48 h it is above the background induction levels of SaI and SaI/A^ktr tumor cells (see Table III for SaI and SaI/A^ktr values). Truncation of either the α- or β-chain therefore diminishes B7 induction, suggesting that the cytoplasmic domain of the class II heterodimer is involved in B7-1 and B7-2 expression.

SaI/A^k tumor cells with truncated α- or β-chains are immunogenic in A/J mice

Since our previous studies (3) and the results of this report suggest that B7-1 and/or B7-2 expression is necessary for tumor rejection, we have also assessed the immunogenicity of the SaI/$A^k\alpha$tr and SaI/$A^k\beta$tr tumor cells. As shown in Table VI, 3/4 of the SaI/$A^k\alpha$tr and 2/2 of the SaI/$A^k\beta$tr clones are rejected by autologous A/J mice, while 1/4 of the SaI/$A^k\alpha$tr clones gives rise to progressively growing tumor. SaI/A^ktr tumor cells having both α- and β-chains truncated also give rise to progressively growing tumor, while SaI cells with full-length, wild-type class II molecules (SaI/A^k) are rejected. The SaI/$A^k\alpha$tr 8.2 clone that is malignant appears to be an anomaly, since three other SaI/$A^k\alpha$tr clones are rejected. In most cases, truncation of either the α- or β-chain therefore does not negate the protective effect of MHC class II Ag expression, while truncation of both chains causes reversion to a tumorigenic phenotype.

Table VI. *Tumorigenicity of SaI sarcoma cells transfected with wild-type, truncated, or mutated MHC class II genes*[a]

Tumor Cells	Tumor Incidence
SaI/A^k	0/5
SaI	5/5
SaI/A^ktr	5/5
SaI/$A^k\alpha$tr 8.7	0/10
SaI/$A^k\alpha$tr 8.12	0/5
SaI/$A^k\alpha$tr 8.2	5/5
SaI/$A^k\alpha$tr 8.26	0/12
SaI/$A^k\beta$tr 12.11	0/5
SaI/$A^k\beta$tr 12.10	0/5

[a] Autologous A/J mice were challenged i.p. with 10^6 tumor cells and followed for tumor incidence as described in *Materials and Methods*.

Since the B7 induction levels were so low for SaI/$A^k\alpha$tr cells (see Table V), we confirmed the requirement for B7 in rejection of these tumor cells by treating the SaI/$A^k\alpha$tr-challenged mice with mAbs to B7-1 and B7-2. Five out of five syngeneic A/J mice treated with mAbs to B7-1 plus B7-2, and subsequently challenged with 10^6 SaI/$A^k\alpha$tr tumor cells, developed tumors, confirming the requirement for B7-1 and/or B7-2 expression for tumor rejection. Rejection of SaI/$A^k\alpha$tr tumor is therefore dependent on expression of B7 molecules, as is rejection of all of the other immunogenic SaI transfectants.

Discussion

B7-1 is a well characterized molecule that has a clearly defined costimulatory function in the activation of Th cells (reviewed in Ref. 20). B7-2, a more recently identified molecule (9, 21–26), is also a costimulatory factor. Recent studies in nontumor systems indicate that B7-1 and B7-2 expression may stimulate qualitatively different immune responses. In at least two model autoimmune diseases (experimental autoimmune encephalomyelitis and insulin-dependent diabetes), expression of B7-2 down-regulates autoimmunity by preferentially facilitating the development of Th2 cells instead of disease-producing Th1 cells (27, 28). Indeed, experiments in several nontumor systems suggest that B7-1 is a costimulatory signal for Th1 cells, while B7-2 is a signal for Th2 cells. As a result, expression of B7-2 and subsequent activation of Th2 cells skews immunity away from a cell-mediated response and thereby down-regulates cellular autoimmunity (reviewed in Ref. 29). In antitumor responses, one would like to amplify the Th1 response and thereby increase cell-mediated immunity to autologous tumor. If B7- 2 functions similarly in tumor responses, as it appears to in at least some autoimmune responses, one would predict that expression of B7-2 would be detrimental to the antitumor immune response. In the tumor system described in this report,

however, tumor cell expression of B7-2 enhances tumor immunogenicity, both with and without accompanying MHC class II expression, thereby resulting in tumor rejection. These results with B7-2 are very similar to our previously reported results with B7-1 (3, 4) and demonstrate that B7-2 facilitates tumor immunity just as well as tumor cell expression of B7-1. Parallel studies by Chen and colleagues (19) note similar effects of B7-2 expression for a mouse mastocytoma tumor. Contrary to results in nontumor systems, expression of B7-2 by tumors therefore enhances tumor cell immunogenicity and induces a protective immune response.

In addition, several Th2-type cytokines (e.g., IL-4, IL-6, and IL-10) have been shown in transfection studies (reviewed in Ref. 30) to enhance tumor rejection, thereby supporting the concept that induction of Th2 cells can facilitate tumor rejection.

Results from several labs have demonstrated that tumor cell expression of B7 genes can enhance tumor rejection of some tumors, but not others (1–4, 31). In some cases, these differences can be ascribed to co-expression of MHC class I vs MHC class II molecules. For example, co-expression of MHC class II plus B7-1 molecules by the highly malignant, poorly immunogenic B16 melanoma significantly reduces the metastatic potential of this tumor, while expression of B7-1 without accompanying MHC class II expression does not significantly alter the malignant phenotype (V. K. Clements and S. Ostrand-Rosenberg, unpublished results). Apparent differences in tumorigenicity following B7 transfection may, therefore, be either the result of co-expression of MHC class I and/or MHC class II molecules, or alternatively, it may be due to tissue origin, the innate immunogenicity of the tumor cells (as suggested in Ref. 5), or other undefined factors.

In previous studies with the SaI/A^k tumor, both $CD4^+$ and $CD8^+$ T cells have been shown to be required for tumor rejection, although only $CD4^+$ T cells are required for immunization of naive mice against subsequent challenge with wild-type tumor (4; and V. K. Clements and S. Ostrand-Rosenberg, unpublished observations). If the induced B7 molecules are delivering costimulatory signals, then it is likely that these signals are being delivered directly to the relevant $CD4^+$ and/or $CD8^+$ T cells, suggesting that the genetically modified tumor cells are functioning directly as APC for tumor peptides. Several immunotherapeutic approaches were designed around the hypothesis that an appropriately modified tumor cell could function as an APC (1–3); however, this hypothesis is controversial, and the precise mechanism of improved tumor rejection is unclear (reviewed in Ref. 6). Indeed, two recent studies (31, 32) using B7-1-transfected class I^+II^- tumors indicated that B7-1 was a target molecule for effector cells, rather than acting as a second signal, making it unlikely that the tumor cell functioned directly as an APC. In other studies (4), we reported that therapeutically effective immunity was induced only when the MHC class II and the B7 signals were delivered by the same tumor cell. This result is difficult to interpret unless the tumor cell itself is the APC. The apparent discrepancy between these results may be due to differences in Ag presentation by class I molecules (31, 32) vs class II molecules (our studies; 3, 6–8), since our studies target the activation of $CD4^+$ T cells by class II^+ APC, while other studies target the activation of $CD8^+$ T cells by class I^+ APC. Despite the differences, the studies presented in this report documenting induction of B7-1 and B7-2 molecules on tumor cells expressing wild-type class II molecules support the contention that the tumor cell itself expresses the costimulatory molecules. It is therefore feasible that the tumor cell could be an APC during the immunization process. Although our experiments to date strongly suggest that the modified tumor cells are APC for tumor peptides, experiments identifying the relevant APC following immunization with class II^+B7^+ tumor cells are necessary to conclusively demonstrate such a role.

Although tumor cell expression of B7-1 and/or B7-2 genes via gene transfection has been shown to enhance tumor rejection, it has not been clear up to now whether expression of B7 molecules occurs during tumor rejection of non-B7-transfected tumor. The Ab blocking studies of Table I show an absolute requirement for B7 expression, while the induction studies of Table III demonstrate a strong correlation between B7 expression and tumor rejection. Recent experiments in which class $II^+B7\text{-}1^+$ SaI transfectants were shown to be superior immunotherapeutic agents for the treatment of established sarcomas, as compared with tumor cells expressing either molecule alone, further confirm the requirement for B7 expression during immune-mediated tumor rejection. Expression of B7 molecules therefore has a strong correlation with immune-mediated tumor rejection, even in non-B7-transfected cells, and it is therefore likely that rejection of immunogenic tumor involves costimulation via B7. Whether the low level of $B7^+$ cells in the tumor cell bit maps of SaI and SaI/A^ktr cells (see Table III) is due to B7 expression by tumor cells or to contamination with $B7^+$ host cells (e.g., macrophages) is unclear. Regardless of the identity of the B7-expressing cells, this low level expression is clearly not sufficient to facilitate tumor rejection, since SaI and SaI/A^ktr cells are highly malignant.

Although the precise mechanism of induction of tumor cell-encoded B7 expression is not clear, the process appears to involve the cytoplasmic domain of the class II heterodimer, since tumor cells expressing class II molecules truncated for most of their cytoplasmic domain do not express B7. Although each chain appears capable of mediating B7 induction to some degree, maximum induction occurs when both the α- and β-chains are full length. These results are in agreement with the studies of Nabavi et al. (11) in which the class II cytoplasmic region was shown in an in vitro system to mediate up-regulation of B7. These observations are also most consistent with an induction mechanism involving intracellular signaling events initiated by the cytoplasmic region of the class II heterodimer.

Since not all tumors become immunogenic when transfected with B7 genes, expression of B7 is clearly not universally necessary or sufficient for improving tumor cell immunogenicity. Given the requirement for costimulation, however, it is feasible that alternative costimulatory and/or activation molecules other than B7 family members may enhance responses to those tumors that are not responsive to B7 expression.

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Reduction of Established Spontaneous Mammary Carcinoma Metastases following Immunotherapy with Major Histocompatibility Complex Class II and B7.1 Cell-based Tumor Vaccines[1]

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ABSTRACT

For many cancer patients, removal of primary tumor is curative; however, if metastatic lesions exist and are not responsive to treatment, survival is limited. Although immunotherapy is actively being tested in animal models against primary tumors and experimental metastases (i.v. induced), very few studies have examined immunotherapy of spontaneous, established metastatic disease. The shortage of such studies can be attributed to the paucity of adequate animal models and to the concern that multiple metastatic lesions may be more resistant to immunotherapy than a localized primary tumor. Here, we use the BALB/c-derived mouse mammary carcinoma, 4T1, and show that this tumor very closely models human breast cancer in its immunogenicity, metastatic properties, and growth characteristics. Therapy studies demonstrate that treatment of mice with established primary and metastatic disease with MHC class II and B7.1-transfected tumor cells reduces or eliminates established spontaneous metastases but has no impact on primary tumor growth. These studies indicate that cell-based vaccines targeting the activation of $CD4^+$ and $CD8^+$ T cells may be effective agents for the treatment of malignancies, such as breast cancer, where the primary tumor is curable by conventional methods, but metastatic lesions remain refractile to current treatment modalities.

INTRODUCTION

In human breast cancer, if metastases are not present, surgical removal of the primary tumor can lead to full recovery of the patient. However, if the primary tumor has metastasized, then other therapies such as hormone therapy (1), chemotherapy (2, 3), and/or radiation therapy (4) are used to eliminate metastatic cells. In many cases, these conventional treatments only lead to temporary control of the disease and provide only an average 3-year survival rate postdiagnosis (5). More effective therapies are clearly necessary for treating metastatic disease. Immunologists have recently proposed and tested a variety of novel strategies for generating cell-based tumor vaccines, and these approaches hold promise for additional treatment modalities. These approaches have focused on the stimulation of $CD8^+$ CTLs because these effector cells are capable of specifically and directly destroying malignant tumor cells. For example, various cytokine genes and/or surface molecules have been transfected into tumors, and the modified tumor cells have been used as cell-based vaccines to enhance antitumor immune responses (reviewed in Refs. 6 and 7). Although some of these studies were designed to circumvent the need for $CD4^+$ T_h[3] lymphocytes by allowing the tumor cells to directly supply cytokines to CTLs (6), other studies were directly aimed at increasing T_h cell generation (8, 9). Both approaches demonstrated that optimal immunity required both $CD4^+$ and $CD8^+$ T cells (8–12). Most of these studies have focused on the treatment of primary tumors, and only a limited number have addressed experimental metastases (*e.g.*, Refs. 13–16). Although even fewer groups focused on established spontaneous metastatic disease, those studies used either severe combined immunodeficient mice or anatomically incorrect tumor challenges in the footpad (17–19). Effective therapies for distant metastatic cells, therefore, have not been extensively studied and remain elusive.

T cells recognize antigen (peptide)/MHCs through their T-cell antigen receptor (20). However, to achieve maximum activation of $CD4^+$ or $CD8^+$ T-cells, a second T-cell antigen receptor-independent signal (costimulation) is required (21). Numerous studies have demonstrated the role of B7.1 and B7.2 in costimulation (22). Other molecules, such as intercellular adhesion molecule-1, VCAM-1, heat-stable antigen, and 4-1BB ligand have also been shown to function in a costimulatory role (23–27). Previously, we demonstrated that the transfection of MHC class II genes into mouse sarcoma and melanoma cells enhanced primary tumor rejection and reduced experimental (i.v.) metastases, respectively (8). Furthermore, expression of either B7.1 or B7.2 in addition to MHC class II increased these effects (8, 9). Not surprisingly, these responses were dependent on both $CD4^+$ and $CD8^+$ T cells. We now propose that by designing tumor cells as vaccination vehicles for stimulating both $CD4^+$ and $CD8^+$ T-cells, it should be possible to induce tumor-specific immunity to treat spontaneous metastatic disease.

To test this hypothesis, we have used the poorly immunogenic BALB/c mouse-derived 4T1 mammary carcinoma (28–30). This tumor shares many characteristics with human mammary cancers, making it an excellent animal model, and it expresses adequate levels of MHC class I molecules, making it a suitable target for $CD8^+$ T cells. Because 4T1 is 6-thioguanine resistant, micrometastatic cells can readily be detected at very early stages of growth, allowing us to quantitatively monitor the effects of the immunotherapy approach on spontaneous metastasis development.

MATERIALS AND METHODS

Animals and Reagents. Female BALB/c and BALB/c *nu/nu* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and/or bred in the University of Maryland Baltimore County animal facility and were used at 8 weeks of age. Reagents were purchased as indicated: Lipofectin and G-418 sulfate (Geneticin; Life Technologies, Inc., Gaithersburg, MD); collagenase types 1 and 4 (Worthington Biochemical Corp., Freehold, NJ); elastase (ICN, Costa Mesa, CA); hyaluronidase, BSA, 6-thioguanine (2-amino-6-mercaptopurine), and methylene blue, Sigma Chemical Co. (St. Louis, MO).

cDNA Expression Vectors. The expression vector pHβ-Apr-1-neo has been described previously (31). Using PCR, cDNAs encoding the $A_\alpha{}^d$ and $A_\beta{}^d$ class II MHC genes were amplified from RNA isolated from A20 B-lymphoma cell line. Primers for the $A_\alpha{}^d$ chain (sense, 5′-CTCCGCGAGTCGACGATGCCGTGCAGCAGA-3′; and antisense, 5′-ACAGCGGATCCTCATAAAGGCCCTG-3′) and $A_\beta{}^d$ chain (sense, 5′-CCTGTGCAGTCGACATGGCTCTGCAGAT-3′; and antisense, 5′-GACACGGATCCTCACTGCAG GAGCC-3′) incorporated a *Sal*I site at their 5′ end and a *Bam*HI site at their 3′ end for

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[3] The abbreviations used are: T_h, T-helper; TD, mean tumor diameter; LN, lymph node; CC, correlation coefficient; NK, natural killer; APC, antigen-presenting cell; mAb, monoclonal antibody.

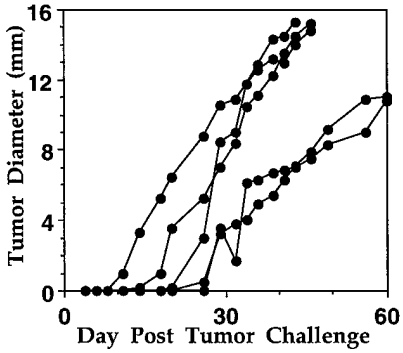

Fig. 1. 4T1 cells are highly tumorigenic. Syngeneic BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells. Primary tumors were measured every 3–4 days, and the mean TD was calculated as described in "Materials and Methods." *Lines*, individual mice.

subcloning into the parental vector. The expression vector containing the B7.1 cDNA was also generated using PCR and was described previously (32). The final constructs contained only the sequence within the coding region for each cDNA and conferred resistance to G-418.

Cell Lines and Transfectants. 4T1, a 6-thioguanine-resistant cell line derived from a BALB/c spontaneous mammary carcinoma, was kindly supplied by Dr. Fred R. Miller (Michigan Cancer Foundation, Detroit, MI; Ref. 30). Unmodified tumor cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine product (Hyclone, Logan, UT) and 1× antibiotic-antimycotic (Life Technologies, Inc.). Transfectants were made to express either MHC class II or B7.1 by using Lipofectin according to the manufacturer's instructions. Cells were selected with 400 μg/ml G-418, cloned by limiting dilution 48 h after transfection, stained for surface antigen expression, and analyzed by flow cytometry as described previously (8, 9). The following antibodies were used: 34-5-8, mouse anti-H-$2D^d$ (33); 16.3.1, mouse anti-H-$2K^k$ (34); MKD6, mouse anti-I-A^d (35); 3JP, mouse anti-I-$A^{b,k}$ (36); and 1G10, rat anti-B7.1 (37).

***In Vivo* Tumor Growth.** Mice were challenged s.c. in the abdominal mammary gland with either parental or transfected 4T1 tumor cells. Primary tumors were measured every 3 or 4 days following tumor challenge using vernier calipers. Mean TD was calculated as the square root of the product of two perpendicular diameters. Animals were sacrificed when the TD reached 14–16 mm or when the mice became moribund, according to University of Maryland Baltimore County Institutional Animal Care and Use Committee guidelines.

Spontaneous Metastases Assay. Spontaneous metastases were measured by adapting methods described previously by Aslakson and Miller (30). Mice were challenged s.c. in the abdominal mammary gland with 5×10^3 parental or transfected 4T1 tumor cells and sacrificed at the times indicated. Several organs were removed from each mouse, uniquely identified, and further prepared as follows: Blood and draining LNs were prepared as described previously (30). Liver samples were finely minced and digested in 5 ml of enzyme cocktail containing 1× PBS, 0.01% BSA, 1 mg/ml hyaluronidase, and 1 mg/ml collagenase type 1 for 20 min at 37°C on a platform rocker. Lung samples were finely minced and digested in 5 ml of enzyme cocktail containing 1× PBS, 1 mg/ml collagenase type 4 and 6 units/ml elastase for 1 h at 4°C on a rotating wheel. Brain samples were finely minced and digested for 2 h at 37°C on a platform rocker with 5 ml of the same enzyme cocktail used for lung samples. After incubation, all samples were filtered through 70-μm nylon cell strainers and washed two to three times with 1× HBSS. Resulting cells were resuspended and plated neat or serially diluted in 10-cm tissue culture dishes in medium containing 60 μM thioguanine for clonogenic growth. 6-Thioguanine-resistant tumor cells formed foci within 10–14 days, at which time they were fixed with methanol and stained with 0.03% methylene blue for counting. Clonogenic metastases were calculated on a per-organ basis.

Statistical Analyses. A Student's *t* test for unequal variances was performed using Microsoft Excel Version 5.0 to determine the statistical significance of indicated data.

RESULTS

Inoculation of Small Quantities of 4T1 Mammary Carcinoma Induces Primary Tumor Formation and Spontaneous Metastatic Disease in Syngeneic BALB/c Mice. Previous studies by Miller and colleagues (29, 30) and others (28) established that the 4T1 mammary carcinoma is highly tumorigenic and spontaneously metastatic in syngeneic BALB/c mice. Because we are developing immunotherapy strategies for the treatment of metastatic malignancies, we have confirmed these results and assessed metastatic disease in additional target organs as a prelude to our therapeutic studies. As shown in Fig. 1 and Table 1, primary tumors form in 100% of BALB/c mice when as few as 5×10^3 cells are injected s.c. in the abdominal mammary gland. These tumors are palpable within 11–26 days after injection and reach 14–16 mm in TD within 40–69 days. At higher doses ($>10^4$), primary tumors develop more rapidly, as reflected in a shortened tumor onset and decreased survival time. Although inoculation of lower doses of 4T1 (10^3) also induces primary tumor formation, the tumor incidence decreases to 60% of inoculated mice. The 4T1 tumor, therefore, is highly tumorigenic, even at relatively low doses of inoculating cells.

To confirm the metastatic potential of the 4T1 mammary carcinoma, female BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 4T1 cells, and metastasis formation was assessed. Mice were sacrificed at varying times after inoculation and the kinetics of spontaneous metastasis formation were assessed in the draining LN, lung, liver, blood, and brain by plating out dissociated organs in medium supplemented with 6-thioguanine. Because 4T1 cells are 6-thioguanine resistant, individual tumor cells form foci in culture, each focus representing an individual clonogenic tumor cell. The number of foci, therefore, is a direct measure of the number of metastatic tumor cells per organ, and the *in vitro* amplification allows for the quantitation of micrometastatic tumor cells, which would otherwise not be detectable.

Table 2 shows the distribution and subsequent spread of metastatic tumor cells in the various organs at progressive times after inoculation. For example, at day 14 or 18 after primary s.c. inoculation, distant spontaneous metastases were measurable in the LN of 11 of 12 mice and the lungs of 13 of 13 mice. By day 22, the livers of three of five mice had clonogenic metastases, whereas the blood of only one of eight mice contained tumor cells. Because only a portion of the blood was recovered, this value may be an underestimate. By week 4, the blood, liver, and lungs of 75–100% of mice contained tumor cells. Some of the organs with clonogenic tumor cells showed visible metastatic lesions; however, many of the organs appeared phenotypically normal and showed no visible signs of tumor. Also by week 4, the draining LN of five of eight mice had been engulfed by the primary tumor and, thus, could not be tested. Metastatic cells in the brain were first detected at week 5 (27% of mice) and the frequency of mice with metastatic cells in the brain increased (67%) as time progressed. Metastases in the blood, LN, liver, and/or brain of indi-

Table 1 *Tumor growth analysis of 4T1 mammary carcinoma in syngeneic BALB/c mice*

BALB/c mice (five mice/group) were challenged s.c. in the abdominal mammary gland with the indicated number of parental 4T1 tumor cells. The tumor incidence is the number of animals that developed progressive tumors. As described in "Materials and Methods," animals that developed tumors were sacrificed when the TD reached 14–16 mm or when the mice became moribund.

Challenge dose	Tumor incidence	Tumor onset (days)	Time to sacrifice (days)
1×10^3	3/5	15–20	45–61
5×10^3	5/5	11–26	40–69
1×10^4	5/5	8–10	35–46
1×10^5	5/5	6–8	35
1×10^6	5/5	4–7	30

Table 2 *4T1 mammary carcinoma cells spontaneously metastasize in BALB/c mice*

BALB/c mice were challenged s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 tumor cells. Mice were sacrificed at various times after tumor challenge, and the draining lymph node, lung, liver, blood, and brain tissues were removed. Each organ was individually prepared as described in "Materials and Methods" and plated for metastatic cell outgrowth. Data indicate the number of animals positive for spontaneous metastases of the total number tested for each organ. The numbers in parentheses show the range of clonogenic metastases found in the positive organs.

	Spontaneous metastases				
Harvest day	LN	Lung	Liver	Blood	Brain
14–18	11/12 (2–57)	13/13 (1–43)	0/11	0/13	ND[a]
22	7/9 (5–35)	6/11 (32–338)	3/5 (1)	1/8 (1)	ND
30–32	2/3 (15–83)	10/10 (6–116,500)	7/8 (7–3,700)	3/4 (6–82)	ND
34–37	ND	10/12 (315–267,000)	11/14 (32–7,800)	5/11 (1–24)	3/11 (1–116)
>42	ND	14/14 (1,109–200,000)	6/8 (1,100–12,200)	6/8 (25–490)	4/6 (5–613)

[a] ND, not done.

vidual mice were only present when the individual contained lung metastases and not *vice versa*. The pathway of metastasis for the 4T1 tumor, therefore, appears to be from the primary tumor to the lungs and the draining LN and, subsequently, to the liver, blood, and brain.

There is frequently a correlation in human disease between the size of primary tumor and extent of metastatic disease. To determine whether this observation is modeled by the 4T1 tumor, the number of clonogenic tumor cells in the lung, liver, blood, LN, and brain has been plotted as a function of the TD at the time of harvest. As shown in Fig. 2*A*, there is a positive correlation (CC = 0.684) between size of primary tumor at time of sacrifice and the number of clonogenic lung metastases. Similar correlations between TD at the time of harvest and clonogenic metastases were also seen for liver (Fig. 2*B*, CC = 0.520), blood (Fig. 2*C*, CC = 0.396), and brain (Fig. 2*D*, CC = 0.426). No correlation was seen between the number of clonogenic metastases in LN and the size of primary tumor (Fig. 2*E*, CC = 0.134) because the number of samples were limiting. The 4T1 tumor, therefore, shows a pattern of metastatic spread comparable to human mammary carcinoma, and assessment of lung metastases best approximates the extent of metastatic disease in tumor-bearing mice.

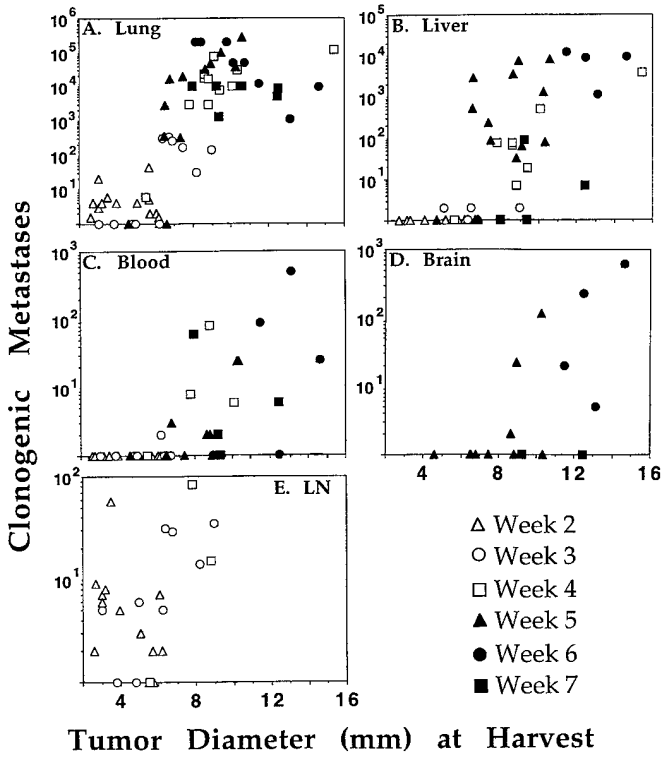

Fig. 2. 4T1 tumor cells spontaneously metastasize to the lungs (*A*), liver (*B*), blood (*C*), brain (*D*), and LN (*E*). Syngeneic BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells. Mice were sacrificed at varying times after inoculation (weeks 2–7), and the number of metastatic tumor cells was determined as described in "Materials and Methods." *Data points*, individual mice.

Expression of MHC Class II or B7.1 by 4T1 Transfectants Reduces Tumorigenicity and Metastatic Potential. In previous studies, we demonstrated that sarcoma cells transfected with syngeneic MHC class II plus B7.1 genes are an effective cell-based vaccine for the treatment of established, primary, solid tumors (9). That strategy was based on the hypothesis that such vaccines could activate both $CD4^+$ and $CD8^+$ tumor-specific T-cells and that optimal activation of $CD8^+$ T-cells requires "help" from $CD4^+$ T-cells. Because such vaccines might be very desirable agents for the treatment of disseminated metastatic disease, we have now extended our studies to the spontaneously metastatic 4T1 breast carcinoma.

4T1 tumor cells were transfected with plasmids containing MHC class II, B7.1, and/or the selectable neomycin resistance genes. Following limiting dilution cloning, several clones were chosen based on their surface expression of MHC class I, class II, and B7.1, as detected by indirect immunofluorescence staining (Fig. 3). All transfectants express similar levels of MHC class I as compared to parental 4T1 cells (Fig. 3, *a–h*). Two of the MHC class II transfectant clones (4T1/A^d-12 and 4T1/A^d-30) express similar levels of MHC class II, whereas the third class II transfectant (4T1/A^d-1) expresses higher levels (Fig. 3, *j–l*). Of the four B7.1 transfectants, two clones (4T1/B7.1-1 and 4T1/B7.1-6) express similar levels of B7.1, which are slightly higher than the levels expressed by the two other transfectants (4T1/B7.1-15 and 4T1/B7.1-23 (Fig. 3, *u–x*). 4T1 cells transfected with the empty parental vector (4T1/neo) do not express either MHC class II or B7.1 (data not shown), as observed with untransfected 4T1 cells (Fig. 3, *i* and *q*).

To test the immunogenicity and tumorigenicity of the class II and B7.1 transfectants, syngeneic female BALB/c mice were challenged in the abdominal mammary gland with 5×10^3 tumor cells, and the challenged mice were followed for primary tumor growth and metastasis formation. Fig. 4 shows the number of clonogenic tumor cells in the lungs *versus* TD at time of sacrifice (*A–H*), and the growth rate of the primary tumor (*A–H, insets*) for the various transfectants. With the exception of 4T1/A^d-30 (Fig. 4*D*, *inset*), all of the transfectants show some reduction in primary tumor growth rate and/or lack of tumorigenicity, although only the 4T1/A^d-12 transfectant does not form primary tumors in any of the inoculated mice (Fig. 4*C*). In contrast, the metastatic potential of both the class II^+ and $B7.1^+$ transfectants is markedly reduced relative to 4T1 cells. For example, 17 of 21 mice inoculated with class II^+ transfectants contained <5,000 metastatic cells in the lung (Fig. 4, *B–D*), whereas 15 of 15 mice inoculated with wild-type 4T1 cells have 5,000–120,000 metastatic cells in the lung (Fig. 4*A*). For the $B7.1^+$ transfectants, 19 of 20 inoculated mice contained 0–432 metastatic cells, with only one mouse displaying >10,000 tumor cells in the lungs (Fig. 4, *E–H*). Primary tumor growth in immunocompetent syngeneic mice, therefore, is inconsistently re-

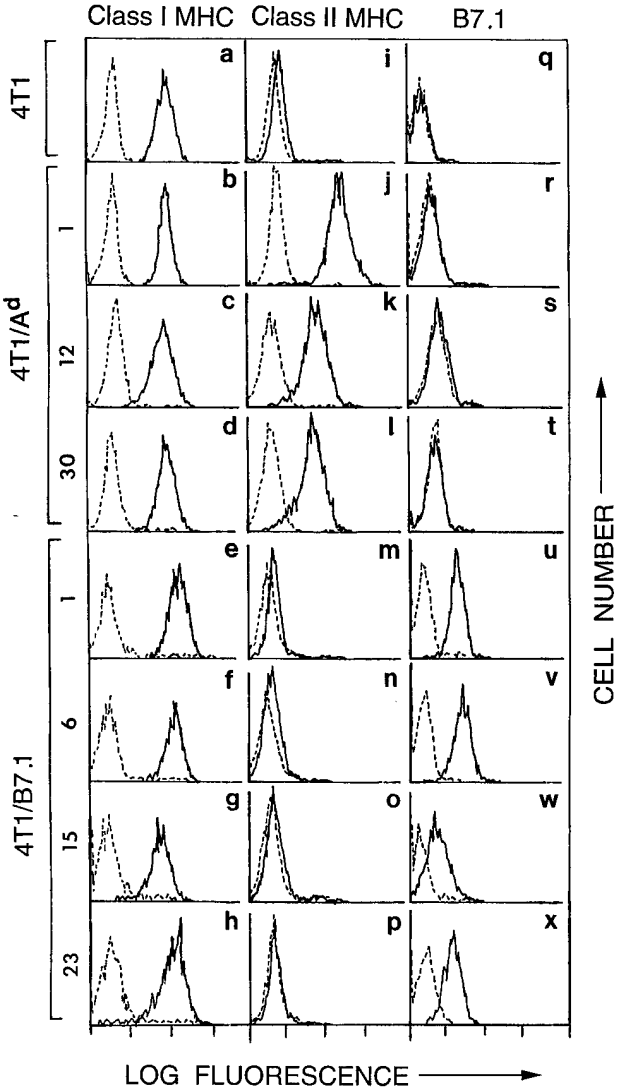

Fig. 3. 4T1 mammary carcinoma transfectants express either I-A^d class II MHC or B7.1 molecules. Parental 4T1 cells and transfectants were stained by indirect immunofluorescence as described in "Materials and Methods." Class I MHC expression (*a–h*) was measured using the mouse anti-H-$2D^d$ mAb 34-5-8 (——) and irrelevant control mouse anti-H-$2K^k$ mAb 16.3.1 (·····). Class II MHC expression (*i–p*) was measured using the mouse anti-A^d mAb MKD6 (——) and the isotype-matched irrelevant control mouse anti-$A^{b,k}$ mAb 3JP (·····). B7.1 expression (*q–x*) was measured using the rat anti-B7.1 mAb 1G10 (——) with the conjugate alone (·····) as control. The *X* axis shows four logarithmic cycles of fluorescence intensity.

duced by expression of MHC class II or B7.1 genes; however, metastatic potential is reproducibly decreased.

Primary Tumor Growth and Metastasis Formation Are Regulated by T Lymphocytes. To determine whether T cell-mediated immunity is involved in the reduced tumorigenicity and metastatic spread of the class II^+ and $B7.1^+$ transfectants, T cell-deficient *nu/nu* mice were tumor-challenged (5×10^3 cells) and followed for primary tumor growth and metastasis formation. Two MHC class II transfectants and two B7.1 transfectants were used. As shown in Fig. 5, one of the class II^+ transfectants (4T1/A^d-1; Fig. 5*B*) and one of the $B7.1^+$ transfectants (4T1/B7.1-6; Fig. 5*D*) formed tumors and metastases in nude mice similar to unmodified wild-type 4T1 tumor cells (Fig. 5*A*). In contrast, 4T1/A^d-12 (Fig. 5*C*) and 4T1/B7.1-23 (Fig. 5*E*) lines formed primary tumor comparable to 4T1; however, their metastatic potential was much reduced relative to wild-type 4T1 tumor cells. To analyze the effects of T cells in immunocompetent *versus* T cell-deficient mice, primary tumor incidence in BALB/c and BALB/c *nu/nu* mice were compared. As summarized in Table 3, 87% of the BALB/c *nu/nu versus* 20% of the BALB/c mice developed progressive primary tumor following s.c. challenge. The class II^+ and $B7.1^+$ transfectants, therefore, have different primary growth kinetics and metastasis formation in T cell-deficient nude mice *versus* immunocompetent BALB/c mice, suggesting that T lymphocytes are important effector cells for regulating tumor growth *in vivo*.

Immunization of Naive Mice with 4T1 Transfectants Expressing MHC Class II or B7.1 Protects against Metastatic Disease but not Primary Tumor Growth following Wild-type 4T1 Challenge. The experiments of Figs. 1–5 suggest that the reduced primary tumor and metastasis formation of the class II^+ and $B7.1^+$ transfectants *versus* 4T1 cells is due to increased tumor cell immunogenicity. We, therefore, have tested the transfectants as immunotherapeutic agents. In the first regimen, naive, tumor-free syngeneic BALB/c mice were immunized i.p. with 10^6 irradiated transfectants and challenged s.c. 4 weeks later with 5×10^3 live 4T1 parental cells. Mice were sacrificed 5 weeks after the 4T1 challenge and clonogenic tumor cells measured in the lungs. As shown in Fig. 6, all of the transfectants provided some protection against 4T1 metastasis, with 4T1/A^d-12 and the mixture of 4T1/A^d-12 plus 4T1/B7.1-23 providing the maximum protection (<1400 clonogenic cells in each individual lung), and immunization with wild-type 4T1 providing minimal protection. Clonogenic metastatic cells in the liver and blood were also similarly reduced in the transfectant-treated animals (data not shown). Other organs were not monitored for metastatic cells. However, none of the transfectants significantly reduced the growth of the primary tumor (data not shown). Immunization of naive mice with the class II^+ and/or $B7.1^+$ transfectants significantly protects against spontaneous metastatic disease but does not affect primary tumor growth of wild-type 4T1 tumor.

Treatment of Tumor-bearing Mice with Transfectants Expressing MHC Class II or B7.1 Reduces Established Wild-type Metastatic Disease but Does Not Affect Primary Tumor Growth. To model a more realistic clinical situation and to test the transfectants more rigorously, the therapeutic efficacy of two transfectant clones was further tested in mice against established metastases. BALB/c mice were challenged s.c. with 5×10^3 wild-type 4T1 tumor cells and, starting at either day 9 or 14 after 4T1 challenge, they were given injections of irradiated transfectants (4T1/A^d-12 and/or 4T1/B7.1-6) twice a week until the day of sacrifice, approximately 4 weeks later. At the time of sacrifice, primary TDs of control-treated mice (*i.e.*, mice given irradiated 4T1 cells), 6.8–12.5 mm, were comparable to TDs in transfectant-treated animals, 6.3–13.6 mm. The two-tailed *P* was 0.29 when tumor sizes of mice treated with control cells were compared with those of transfectant-treated mice combined. Lungs were subsequently removed, and the number of clonogenic tumor cells was determined. Because this therapy will be used to treat patients with established tumor, the results of this experiment have been plotted as number of clonogenic cells in the lungs *versus* TD at the start of treatment. As shown in Fig. 7, administration of 4T1/A^d-12, 4T1/B7.1-6, or a mixture of cells significantly reduces the number of lung metastases (Fig. 7, *B–D*) relative to treatment with wild-type 4T1 cells (Fig. 7*A*) when primary TDs at the start of treatment were <4 mm. After transforming the number of clonogenic metastases to logarithmic values and analyzing as described in "Materials and Methods," the two-tailed *P* was 0.008 when control-treated mice were compared with transfectant-treated mice combined. When TDs, however, were >4 mm on the initial treatment day, no significant reduction in primary tumor growth or metastatic cells was seen (data not shown). Metastatic spread, therefore, can be significantly reduced by im-

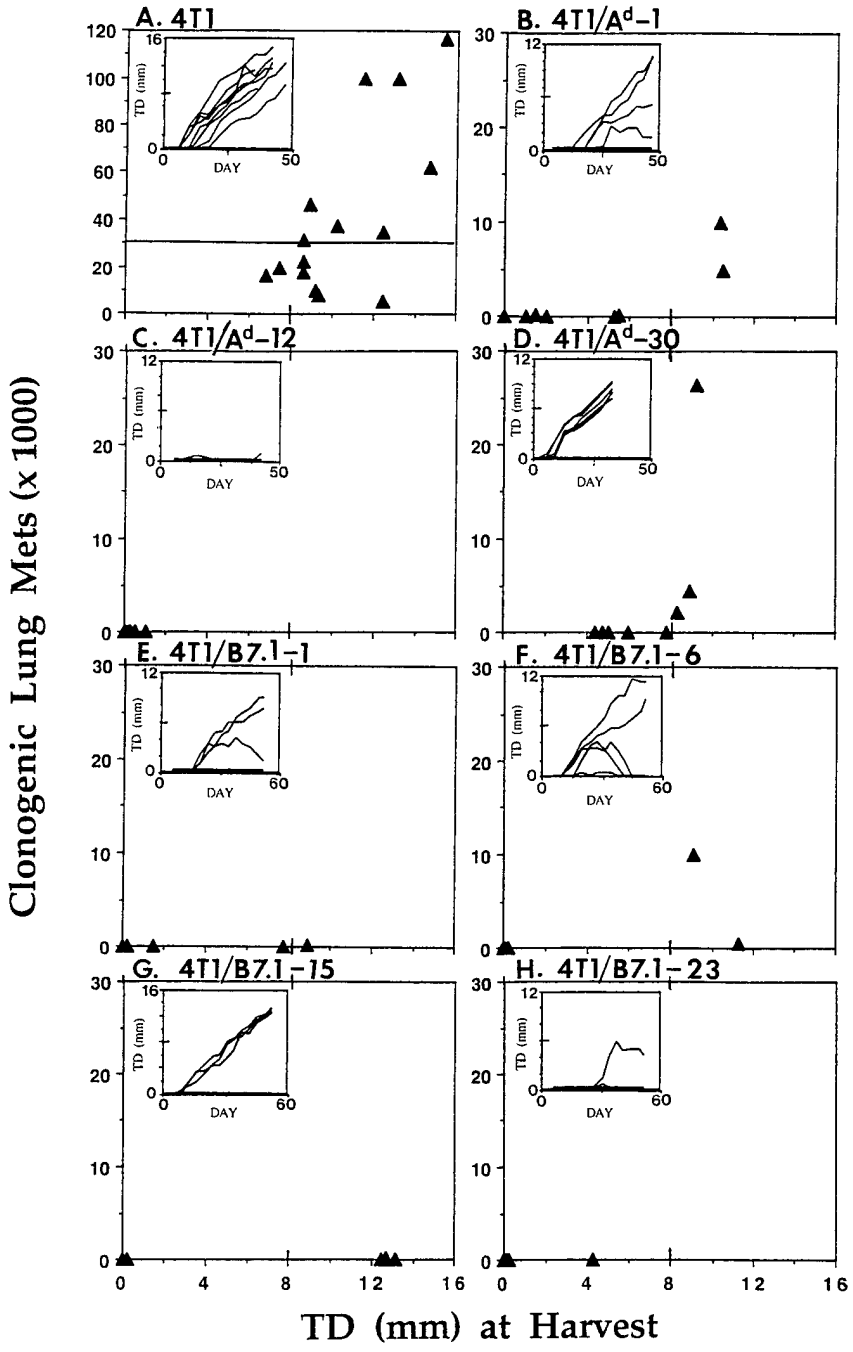

Fig. 4. Expression of either class II MHC or B7.1 reduces metastatic potential and tumorigenicity of the 4T1 transfectants. Female BALB/c mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells (15 mice; *A*), 4T1/A^d-1 (9 mice; *B*), 4T1/A^d-12 (10 mice; *C*), 4T1/A^d-30 (8 mice; *D*), 4T1/B7.1-1 (5 mice; *E*), 4T1/B7.1-6 (5 mice; *F*), 4T1/B7.1-15 (5 mice; *G*), or 4T1/B7.1–23 (5 mice; *H*) and sacrificed 32–55 days later, and the number of metastatic cells in the lungs was determined as described in "Materials and Methods." Primary tumors were measured every 3–4 days. *A–H*, numbers of clonogenic lung metastases (× 1000) *versus* the TD at the time the mice were sacrificed. ▲, individual mice. *Insets*, mean TD (*Y* axis) *versus* days postinoculation (*X* axis). *Lines*, individual mice. Note that the number of clonogenic lung metastases shown on the *Y* axis ranges from 0 to 120 in *A*, as opposed to a range of 0–30 for *B–H*.

munotherapy in mice carrying spontaneously metastatic established tumors, provided treatment originates when the primary tumor is <4 mm in diameter.

DISCUSSION

Many studies during the past 5–10 years have focused on developing immunotherapy strategies for the treatment of solid tumors and have used animal systems to model human disease and to test the efficacy of immunotherapy. Most of these studies have used transplanted primary solid tumors (6, 7) or short-term established experimental (i.v. induced) metastatic cancers, in which therapy was performed very early during metastatic disease (13–16). A small number of studies focused on spontaneous metastases; however, these models used severe combined immunodeficient mice or anatomically incorrect tumor challenge sites (17–19). In many cases, the growth characteristics and kinetics of the model tumors used did not closely follow the natural history of their corresponding human tumor and, hence, were not optimal model systems. In contrast to many mouse tumors, the BALB/c-derived 4T1 mammary tumor, originally derived by Miller and colleagues (29, 30) and others (28), shares many characteristics with its human counterpart mammary carcinoma. For example, 4T1 spontaneously metastasizes while the primary tumor is in place, analogous to human mammary tumors. Sites of metastasis are common between the mouse and human malignancies: spreading first to the lungs and liver in 24–77% and 22–62% of women, respectively, *versus* >95% and >75%, respectively, of BALB/c mice (Table 2; Refs. 38–41). Metastasis to the central nervous system is characteristically less frequent than metastasis to other sites in both

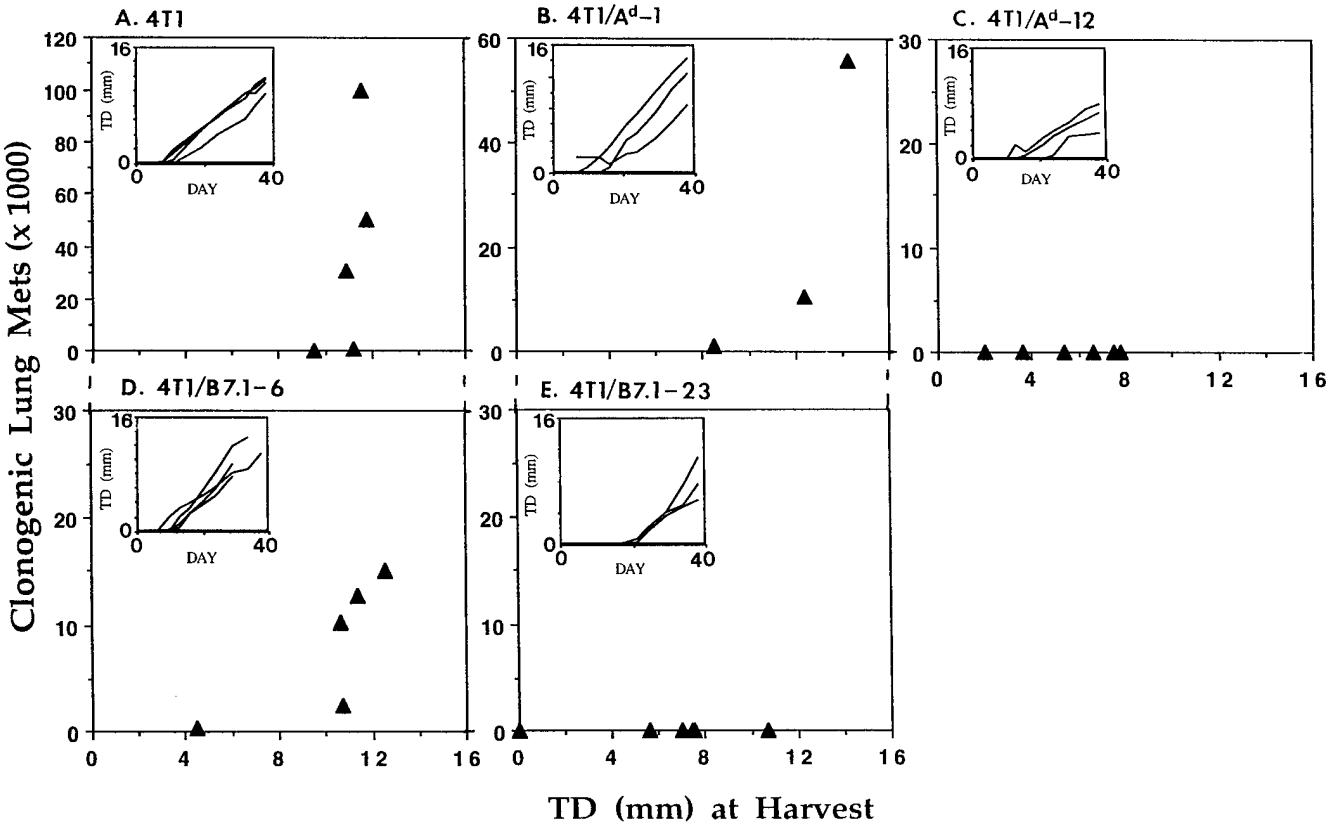

Fig. 5. Different immune effector cells alter primary tumor growth *versus* spontaneous metastasis formation. BALB/c *nu/nu* mice were injected s.c. in the abdominal mammary gland with 5×10^3 parental 4T1 cells (5 mice; *A*), 4T1/A^d-1 (3 mice; *B*), 4T1/A^d-12 (6 mice; *C*), 4T1/B7.1-6 (5 mice; *D*), or 4T1/B7.1-23 (6 mice; *E*), and tumor growth was measured every 3–4 days. Data are plotted as in Fig. 4. Note that the number of clonogenic lung metastases shown on the *Y* axis ranges from 0 to 120 in *A*, as opposed to ranges of 0–60 for *B* and 0–30 for *C–E*.

Table 3 *Tumor incidence of 4T1 transfectants in syngeneic BALB/c versus BALB/c nu/nu mice*

Mice were challenged s.c. in the abdominal mammary gland with 5×10^3 transfected 4T1 tumor cells. The tumor incidence is the number of animals that developed progressive tumors. As described in "Materials and Methods," animals that developed tumors were sacrificed when the TD reached 14–16 mm or when the mice became moribund.

Tumor challenge	Tumor incidence	
	BALB/c	BALB/c *nu/nu*
4T1/A^d-1	3/10	3/3
4T1/A^d-12	1/10	5/6
4T1/B7.1-6	2/5	7/8
4T1/B7.1-23	1/5	5/6

humans and mice (30% and 40%, respectively) and, statistically, occurs later in the disease process (Table 2; Refs. 41 and 42).

In addition to its growth characteristics, the 4T1 tumor has several experimental characteristics that make it an ideal model for testing immunotherapy strategies. A major asset is its stable resistance to 6-thioguanine, enabling the precise quantitation of very small numbers of tumor cells, long before they could be detected visually or accurately quantitated by other methods. Because metastasis to the lungs precedes and always accompanies metastasis to other organs (Table 2), quantitation of lung metastases accurately assesses metastatic disease. The similarity in growth between the 4T1 tumor and human mammary cancer plus the ease of assessing metastatic disease, therefore, make the mouse 4T1 tumor an excellent model for testing potential immunotherapy strategies.

Previous immunotherapy studies using MHC class II and/or B7.1-expressing tumor cells as cell-based vaccines have dealt predominantly with solid, primary tumors (7–9). Here, these vaccines are used for the treatment of metastatic disease. The current studies are also distinct from earlier studies using a variety of cell-based vaccines, including cytokine-transduced/transfected tumor cells, in that spontaneous, established metastases are being treated, rather than short-term experimental (i.v.) metastases. These disease conditions much more closely mimic those of human breast cancer patients, and hence, the

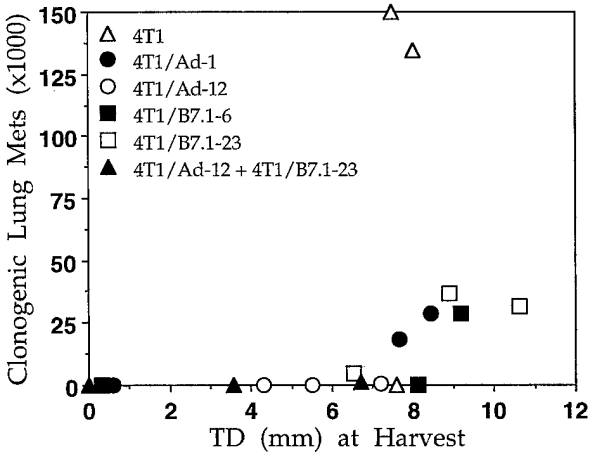

Fig. 6. Immunization with MHC class II$^+$ or B7.1$^+$ transfectants protects naive mice against metastatic disease from parental 4T1 tumor challenge. Syngeneic BALB/c mice (three mice/group) were vaccinated i.p. with 1×10^6 irradiated parental 4T1 cells (△), 4T1/A^d-1 (●), 4T1/A^d-12 (○), 4T1/B7.1-6 (■), 4T1/B7.1-23 (□), or a 1:1 mix of 4T1/A^d-12 plus 4T1/B7.1-23 (▲). Four weeks later, mice were challenged s.c. in the abdominal mammary gland with 5×10^3 live parental 4T1 cells. Five weeks postparental tumor challenge, the TD and the number of clonogenic lung metastases were measured.

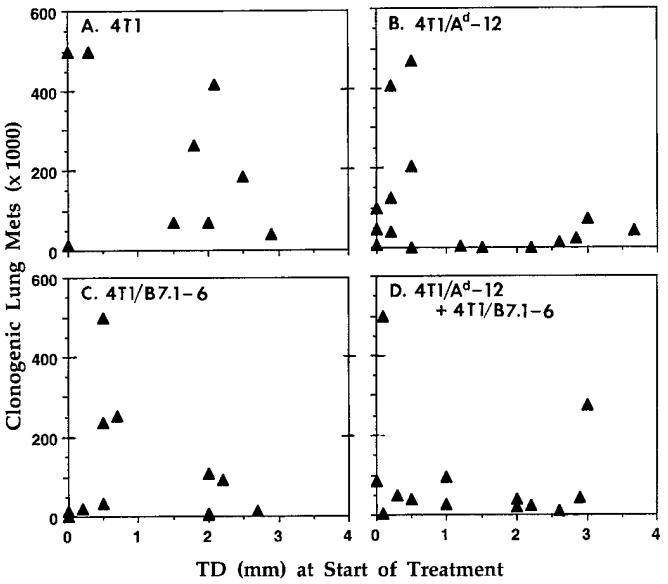

Fig. 7. Immunotherapy of established 4T1 tumors with MHC class II^+ and/or $B7.1^+$ transfectants reduces metastatic disease. Syngeneic BALB/c mice were challenged s.c. in the abdominal mammary gland with 5×10^3 live parental 4T1 cells. At day 9 or 14 postparental tumor challenge, the TD was measured, and the therapeutic injections began. Mice were treated i.p. twice a week until the time of sacrifice with 1×10^6 irradiated parental 4T1 (*A*), 4T1/A^d-12 (*B*), 4T1/B7.1-6 (*C*), or a 1:1 mix of 4T1/A^d-12 plus 4T1/B7.1-6 (*D*) cells. Mice were sacrificed 6 weeks after initial 4T1 tumor challenge, and the number of clonogenic lung metastases was determined. The data are plotted as the TD at the time the therapeutic treatment began *versus* the number of clonogenic lung metastases (×1000) at the time of sacrifice. ▲, individual mice. Statistical analysis was performed using a Student's *t* test for unequal variances as described in the text (two-tailed $P = 0.008$).

observed results may be useful in projecting experimental animal results to human clinical situations.

Treatment of mice carrying 9–14-day established 4T1 tumors with MHC class II and/or B7.1-transfected tumor cells results in a dramatic reduction in the number of metastatic tumor cells relative to mice treated with wild-type 4T1 (Fig. 7), suggesting that such cell-based vaccines may be useful immunotherapeutic agents for the treatment of metastases. The finding that metastatic growth is greatly reduced or eliminated, whereas primary tumor growth is not significantly impacted, is surprising and suggests that immunotherapy may be more useful against metastatic disease than against primary tumor. Because many primary tumors can be successfully surgically resected whereas many metastatic lesions are refractile to current therapy, immunotherapy may have a unique role in cancer treatment.

Because mice with primary tumors with TDs of >2 mm contain LN and lung metastatic cells (Fig. 2), the immunotherapy is limiting proliferation of pre-established metastases. Likewise, because treatment of naive mice produces some animals with no metastases, the immunotherapy is also preventing establishment of new metastases. Therefore, although not routinely curative, this immunotherapy may slow progression of metastatic disease.

Previous therapy studies with B7.1 transfected tumors and primary or experimental metastases indicated that costimulatory molecule expression was effective in vaccines containing "moderately" immunogenic tumor cells but not in vaccines containing "poorly" immunogenic tumor cells (7). By definition, 4T1 cells are poorly immunogenic because immunization of tumor-free mice with irradiated wild-type cells does not provide protective immunity against subsequent challenge with wild-type tumor cells (Figs. 6 and 7). Because immunization with B7.1 transfected tumor cells does not result in reduced primary tumor growth in the immunotherapy protocol, our results agree with these earlier studies (7). However, the finding that B7.1-transfected tumor cells promote significantly reduced metastatic growth in the therapy protocol (Fig. 7) revives B7.1 as a potential candidate for immunotherapy.

The mechanism by which the class II^+ and $B7.1^+$ transfectants are providing their protection is not clear. Because these transfectants displayed varying *in vivo* phenotypes, different types of effector cells may be activated. In most cases, T cells were important in regulating primary tumor growth (Fig. 5); however, their role in outgrowth of metastases is less clear cut. This could easily be explained by an enhancement of nonspecific effectors, such as lymphokine-activated killer cells and/or NK cells, as it has been previously shown that B7.1 can induce NK activity against tumors (32, 43). Alternatively, limiting dilution cloning of the transfectants may have cloned out tumor cells that lost their ability to metastasize (44). Regardless of the *in vitro* and *in vivo* phenotypes of the transfectants (*i.e.*, level of expression of class II and/or B7.1, metastatic potential, and tumorigenicity in BALB/c *versus nu/nu* mice), most clones provide some protection against wild-type metastatic disease (Figs. 6 and 7). Thus, these studies suggest that most transfectants will be useful as vaccines and that cell-based vaccines may be more effective than previously thought.

Transfection of tumor cells with MHC class II plus B7.1 genes was originally designed to produce tumor cells that could directly present antigen to $CD4^+$ T_h cells and $CD8^+$ CTL and, thereby, facilitate optimal antitumor immunity (9, 45). Genetic experiments using bone marrow chimeras and sarcoma tumor cells support this hypothesized mechanism of $CD4^+$ T-cell activation and demonstrate that the genetically modified tumor cells function as the APC for tumor-encoded antigen (46, 47). In contrast, class I-restricted tumor-encoded antigen appear to be presented indirectly via host-derived APCs (48–50). Increased antitumor activity following immunization, therefore, is probably the result of enhanced presentation of tumor antigens and the subsequent activation of multiple helper and effector cell populations.

Why the effectiveness of this treatment is limited to mice with starting tumors with TDs of <4 mm is unclear. Factors such as immunosuppression of tumor-bearing individuals, immunogenicity of tumor antigens, the timing of the developing immune response *versus* outgrowth of the tumor, and involvement of nonspecific effector cell types (*i.e.*, lymphokine-activated killer cells, NK cells, and macrophages) have been discussed at length in the context of other immunotherapy approaches (51–53), and some or all of these factors may be implicated here. Optimal T-cell activation is achieved when B7.1 and MHC class II molecules are expressed by the same APC (9, 54). Our cell-based vaccine, therefore, might be more effective if double transfectants were used rather than the mixture of single transfectants tested in this study. Regardless of the limitations, however, the promising therapeutic responses are encouraging for further testing and development of this approach either alone or in combination with other immunotherapeutic and/or conventional modalities.

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MHC Class II-Transfected Tumor Cells Directly Present Antigen to Tumor-Specific CD4$^+$ T Lymphocytes[1]

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We have developed and shown to be efficacious an immunotherapeutic strategy to enhance the generation of tumor-specific CD4$^+$ T helper lymphocytes. The approach uses autologous tumor cells genetically modified to express syngeneic MHC class II genes as cell-based immunogens and is based on the hypothesis that tumor cells directly present tumor Ags to CD4$^+$ T cells. Since the conventional pathway for CD4$^+$ T cell activation is indirect via professional APC, induction of immunity following immunization with class II-transfected tumor cells was examined in bone marrow chimeric mice. Both tumor and host-derived cells are APC for tumor Ags, suggesting that the efficacy of tumor cell vaccines can be significantly improved by genetic modifications that enhance tumor cell Ag presentation. *The Journal of Immunology,* **1998, 160: 661–666.**

Many of the recently developed strategies for enhancing immunity to autologous tumors involve immunization with genetically modified tumor cells. These approaches are based on the reasoning that tumor cells present potentially immunogenic tumor peptides, and that if modified appropriately, they could directly present tumor Ag and activate T lymphocytes, by-passing the requirement for transfer of tumor Ag (peptide) to host-derived professional APC.

Most of these studies have focussed on the direct activation of tumor-specific CD8$^+$ T lymphocytes, either by providing appropriate costimulatory signals such as B7 (1–4) or by providing cytokines normally produced by CD4$^+$ T helper lymphocytes, such as IL-2 or IL-4 (5, 6). In contrast, we and others have reasoned that optimal tumor-specific CD8$^+$ T cells and long term immune memory will only be generated if sufficient CD4$^+$ Th cells are also stimulated (7–10). As a result, we have targeted the activation of tumor-specific CD4$^+$ T cells. We have hypothesized that class II-transfected tumor cells will present endogenously encoded peptides in the context of the transfected class II molecules and, therefore, directly present tumor peptides to CD4$^+$ T helper lymphocytes. Reports from our laboratory as well as studies by others demonstrate that such genetically modified tumor cells are potent inducers of tumor-specific immunity in naive mice (7, 9, 11) as well as in tumor-bearing mice (12), demonstrating their potential as immunotherapeutic agents. Although these therapeutic results are consistent with the hypothesis that the genetically modified tumor cells directly present Ag to CD4$^+$ T cells during the immunization process, there is no direct evidence demonstrating that the modified tumor cells directly present Ag to responding CD4$^+$ T lymphocytes. Such a pathway would be unconventional, since CD4$^+$ T cells are usually activated by the process of indirect Ag presentation or cross-priming, in which Ag is taken up and presented by host-derived professional APC (i.e., dendritic cells, macrophages, or B lymphocytes) rather than by the Ag-expressing cells themselves (13, 14). Since optimal exploitation of this immunotherapeutic approach will depend on a full understanding of the mechanism through which the genetically modified tumor cells activate T lymphocytes, the present studies were undertaken to identify the cell population(s) that serves as APC during the activation of tumor-specific CD4$^+$ T cells following immunization with class II-transfected tumor cells.

Materials and Methods

Mice

C57BL/6, A/J, and (C57BL/6 × A/J) ((B6 × A/J)F_1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in the University of Maryland Baltimore County animal facility. Bone marrow donors and recipients were female mice, and chimeras were generated as previously described (15). Briefly, recipient (B6 × A/J)F_1 mice were maintained on tetracycline water (0.2%) for 1 wk before and 5 wk after reconstitution and were given gentamicin sulfate s.c. (500 μg) for 7 consecutive days beginning 1 day before irradiation/reconstitution. Approximately 24 h before irradiation/reconstitution, recipients were taken off food. Recipients were irradiated with 875 rad total body irradiation using a ^{137}Cs source (Kewaunee Scientific, Statesville, NC) and reconstituted i.v. with one femur-equivalent of donor bone marrow within 2 to 3 h of irradiation. Chimeras were maintained in a pathogen-free environment for 6 wk before use. All chimeras were tested by indirect immunofluorescence to ascertain bone marrow genotype and assure chimeric status as follows. Concurrent with the in vitro APC assays, spleens of chimeras were removed and stained for MHC class I (H-2K^k for A/J (mAb 16-3-1) (16); H-2K^{b}D^b for C57BL/6 (mAb 28-13-3) (17)) Ags. Positively staining cells were gated relative to conjugate alone controls, and positive cells were compared with wild-type A/J and C57BL/6 splenocytes stained under identical conditions. The percentage of the donor phenotype was calculated by comparing the percentage of positive chimeric spleen cells vs that of wild-type cells. For example, if 98 and 2% of A/J→F_1[3] chimeric splenocytes were stained with the 16-3.1 and 28-13-3 mAbs, respectively, and 99 and 1% of A/J wild-type splenocytes were stained with the 28-13-3 and 16-3.1 mAbs, respectively, then the A/J→F_1 chimeras were considered 99% donor phenotype.

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[3] Abbreviations used in this paper: A/J→F_1, lethally irradiated (C57BL/6 × A/J)F_1 mouse reconstituted with A/J bone marrow; Sal/A^k, Sal tumor cells transfected with A$^k_\alpha$ and A$^k_\beta$ genes; Sal/A^b, Sal tumor cells transfected with A$^b_\alpha$ and A$^b_\beta$ genes; HEL, hen egg lysozyme; Sal/HEL, Sal tumor cells transfected with the hen egg lysozyme gene; B6→F_1, lethally irradiated (C57BL/6 × A/J)F_1 mouse reconstituted with C57BL/6 bone marrow; F_1, (C57BL/6 × A/J)F_1 mouse.

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Cells, transfectants, and hybridomas

The SaI sarcoma and its transfectants were cultured as previously described (7). SaI sarcoma cells transfected with syngeneic MHC class II A^k_α and A^k_β genes (SaI/A^k cells) have been previously described (7). SaI cells expressing I-A^b class II molecules (SaI/A^b cells) were generated by transfecting SaI cells with the A^b_α and A^b_β cDNAs contained in the pKCR3 plasmid (18) plus the pSV2.neo plasmid using the transfection procedure previously described (7). SaI, SaI/A^k, or SaI/A^b cells expressing an endoplasmic reticulum-retained hen egg lysozyme gene (HEL; SaI/HEL, SaI/A^k/HEL, SaI/A^b/HEL cells) were generated as previously described by transfection with the BCMG-HEL plasmid containing the hygromycin^R gene (19). Transfectants expressing MHC class II genes or HEL were maintained in medium supplemented with 400 μg/ml G418 or 400 μg/ml hygromycin, respectively. Double transfectants were maintained on both drugs. 3A9 is an I-A^k-restricted HEL_{46-61}-specific T cell hybridoma (20) and was maintained as previously described (19); JK1290 is an I-A^b-restricted HEL specific hybridoma (21) and was maintained in Iscove's modified Dulbecco's medium supplemented with 10% Fetalclone I (Hyclone, Logan, UT), 1% penicillin, 1% streptomycin, and 1% gentamicin.

Tumor challenges

For tumorigenicity studies, mice were inoculated i.p. with live tumor cells, observed daily for survival, and killed when they became moribund. The inoculation dose was chosen based on previous titration studies (7). For immunization studies, chimeric mice were inoculated i.p. with 5×10^5 to 10^6 live tumor cells and killed 6 or 7 days later. For tumor challenge studies, mice were inoculated i.p. with the indicated number of tumor cells and examined three times per week for tumor growth. Ascites tumors usually became palpable within 10 to 14 days of inoculation and grew progressively. Based on our previous experience with the SaI tumor, if mice do not develop palpable tumor within 2 mo of challenge, they will remain tumor free during their lifetime (7). Tumor incidence is the number of mice with progressively growing tumors divided by the total number of mice challenged. Tumor-bearing mice were killed according to University of Maryland Baltimore County institutional animal care and use committee guidelines when they became moribund. The mean survival time is the time between inoculation and sacrifice.

In vitro Ag presentation assays

Splenocytes from immunized mice were prepared from mechanically dissociated spleens, and B lymphocytes were removed by panning as previously described (22). Resulting T cells (5×10^6 cells/well) were cocultured in flat-bottom 96-well plates with fresh naive A/J or C57BL/6 splenocytes in a final volume of 250 μl/well containing 1 mg/ml lysozyme. Responder to stimulator ratios were 10:1 and/or 50:1. Supernatants were harvested after 24 h and assayed for IL-2 content using ELISA kits as described by the manufacturer (Endogen, Boston, MA). Samples were run in triplicate, and the mean $\pm$ SD determined for each sample. In most cases, SDs were $\leq$5% of test values. Background values (IL-2 release in the absence of HEL) were subtracted from experimental values (IL-2 release in the presence of HEL) to obtain specific IL-2 release. Values were converted to picograms per milliliter using a standard curve incorporated into the IL-2 assay. In some experiments splenocytes were depleted of $CD4^+$ or $CD8^+$ T lymphocytes in vitro (22) or in vivo (12) before use in an APC assay. APC assays using splenocytes from chimeric mice plus T cell hybridomas 3A9 and JK1290 were performed as previously described (19).

Indirect immunofluorescence and flow cytometry

Tumor cells, transfectants, and splenocytes were monitored for cell surface Ag expression by indirect immunofluorescence as previously described (7) and analyzed on an Epics XL flow cytometer (Coulter, Hialeah, FL). The following mAbs were used: I-A^k (10-3-6 or 10-2-16) (23), I-A^b (34-5-3S) (17), K^k (16-3-1) (16), D^d (34-5-8) (24), and lysozyme (HyHEL 7 and 10) (25). Cells monitored for intracellular lysozyme were fixed with paraformaldehyde and stained with a mixture of the HyHEL 7 and 10 mAbs (25) as previously described (19). Isotype controls were performed for surface and cytoplasmically stained cells, and staining was essentially identical with that in fluorescent conjugate alone controls.

Results

In our previous studies the A/J-derived ($H\text{-}2^{k/d}$) SaI sarcoma, when transfected with syngeneic MHC class II A^k_α and A^k_β genes (SaI/A^k tumor cells), induced potent $CD4^+$ T cell-dependent, tumor-specific immunity in syngeneic A/J and semisyngeneic (C57BL/6 $\times$ A/J)F_1 mice. In vitro experiments using HEL-specific, I-A^k-restricted T cell hybridomas showed that SaI/A^k tumor cells transfected with a gene encoding an endoplasmic reticulum-retained HEL (SaI/A^k/HEL tumor cells) present endogenously synthesized HEL peptide to $CD4^+$ T cells (19). The genetically modified tumor cells, therefore, are capable of presenting tumor-encoded Ag directly to T cells. To determine whether during the immunization process the tumor cells themselves are APC for endogenously encoded tumor Ags (direct Ag presentation) or if host-derived cells are the APC (indirect Ag presentation or cross-priming), we used the following genetic approach. (C57BL/6 $\times$ A/J)F_1 mice are lethally irradiated and reconstituted with either C57BL/6 ($H\text{-}2K^bA^bD^b$) or A/J ($H\text{-}2K^kA^kD^d$) bone marrow so that the resulting chimeric mice (B6$\rightarrow F_1$ or A/J$\rightarrow F_1$, respectively) have host-derived APC of either the C57BL/6 or the A/J genotype, respectively. The chimeras are subsequently challenged with either SaI/A^k/HEL or SaI/A^b/HEL tumor cells, respectively, and the MHC restriction pattern of the response is determined. If the tumor cells are the exclusive APC for the tumor-encoded Ag (HEL), then the response will be restricted to the MHC class II genotype of the tumor cells. However, if host-derived cells are the exclusive APC for tumor-encoded HEL, then the T cell response will be restricted to the genotype of the reconstituting bone marrow in the chimeras. If both tumor cells and host cells are APC for tumor-encoded Ags, then the response will be restricted to both the tumor and bone marrow genotypes.

SaI sarcoma cells transfected with MHC class II and/or HEL genes express these gene products at the cell surface or intracellularly

SaI/A^k and SaI/A^k/HEL cells were available from previous experiments (7, 19). SaI/A^b and SaI/A^b/HEL cells were generated by gene transfection as described in *Materials and Methods*. The resulting transfectants were stained for cell surface expression of MHC class II molecules (live cells) or for intracellular expression of lysozyme (paraformaldehyde-fixed and saponin-permeabilized cells). As shown in Figure 1, cells transfected with the MHC class II I-A^k and I-A^b genes expressed comparable levels of these molecules, as measured by staining with the 10-2-16 and 34-5-3S mAbs, respectively (Fig. 1, *i* and *j*, and *e* and *f* for SaI/A^k and SaI/A^b cells, respectively). Similarly, cells transfected with the HEL construct expressed comparable levels of intracellular lysozyme as measured by staining with the mixture of HyHEL 7 and HyHEL 10 mAbs (Fig. 1, *n*, *p*, and *r* for SaI/HEL, SaI/A^k/HEL, and SaI/A^b/HEL cells, respectively.), while untransfected cells were negative for lysozyme (Fig. 1, *m*, *o*, and *q*). HEL transfectants were also stained for cell surface HEL expression and were negative (data not shown). Supernatants of the transfectants were assayed by ELISA for HEL secretion and showed low levels of soluble HEL (1–5 ng/ml/6.7×10^5 cells/24 h). The transfectants were also stained for MHC class I H-$2K^k$, H-$2D^d$, and H-$2L^d$ Ag expression, and these levels were approximately equivalent among all transfectants and parental SaI cells (data not shown).

Lysozyme peptides are presented by both I-A^k and I-A^b MHC class II molecules

Numerous in vitro studies have demonstrated that both I-A^k and I-A^b MHC class II molecules present HEL-derived peptides to $CD4^+$ T cells (21, 26–28). To ascertain that HEL peptides are presented by both class II alleles when the alleles are expressed by SaI sarcoma cells, semisyngeneic (C57BL/6 $\times$ A/J)F_1 mice were challenged i.p. with parental SaI, SaI/A^k, SaI/A^b, SaI/HEL, SaI/A^k/HEL, and SaI/A^b/HEL tumor cells and followed for tumor incidence. As shown in Table I, wild-type SaI and SaI/HEL tumor

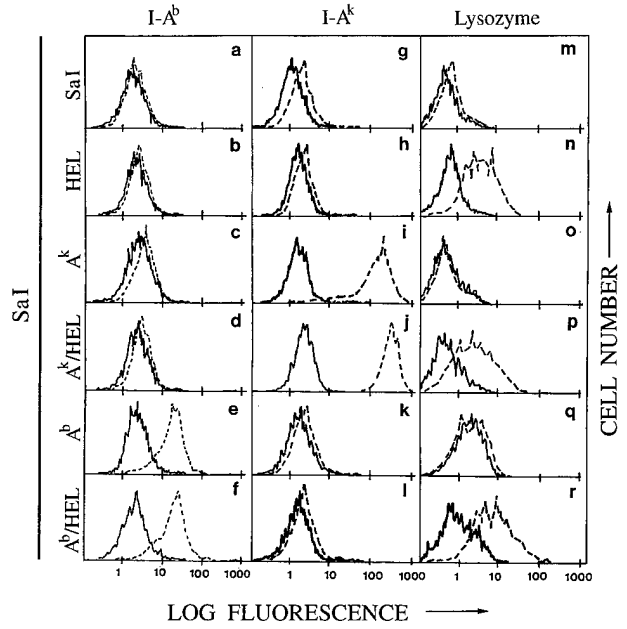

FIGURE 1. Indirect immunofluorescence staining of SaI (*a*, *g*, and *m*), SaI/HEL (*b*, *h*, and *n*), SaI/A^k (*c*, *i*, and *o*), SaI/A^k/HEL (*d*, *j*, and *p*), SaI/A^b (*e*, *k*, and *q*), and SaI/A^b/HEL (*f*, *l*, and *r*) cells. Tumor cells were stained for I-A^b with the 28-13-3 mAb (17) (*a–f*), for I-A^k with the 10-2-16 mAb (23) (*g–l*), or for lysozyme with the HyHEL 7 plus 10 mAbs (25) (*m–r*). MHC class II staining was performed on live tumor cells; lysozyme staining was performed on fixed, permeabilized cells. The second-step, fluorescent conjugate FITC-goat anti-mouse IgG was used with all Abs. The solid line represents staining by fluorescent conjugate alone; the dotted line represents staining by specific Ab plus fluorescent conjugate.

Table I. *MHC class II/HEL-transfected tumor cells are rejected by semisyngeneic (C57BL/6 × A/J)F_1 mice*[a]

Tumor Cells	Tumor Incidence	MST ± SE (days)
SaI	10/17	21 ± 5
SaI/HEL	10/10	21 ± 7
SaI/A^k	10/20	28 ± 13
SaI/A^b	4/10	32 ± 3
SaI/A^k/HEL	0/5	≥ 90
SaI/A^b/HEL	0/5	≥ 90

[a] Semisyngeneic (C57BL/6 × A/J)F_1 mice were challenged i.p. with 10^6 tumor cells and mice were followed for tumor growth.

cells were lethal in ≥94% of F_1 mice, indicating that HEL expression alone does not cause tumor rejection. SaI/A^k and SaI/A^b cells were significantly less lethal than SaI cells; however, they still caused tumors in a significant number of F_1 mice (50 and 40% lethal, respectively). In contrast, SaI/A^k/HEL and SaI/A^b/HEL tumor cells were rejected by 100% of F_1 mice, suggesting that HEL peptides are presented by the I-A^k and I-A^b MHC class II molecules of the tumor cells and function as nominal Ag for T cell recognition.

Immunization of chimeric mice with tumor cell transfectants generates T cells restricted to both the tumor genotype and the host genotype

If the genetically modified tumor cells are the exclusive APC for tumor Ags, then A→F_1 bone marrow chimeras immunized with B tumor cells will have HEL-specific T cells restricted to the tumor (B) MHC genotype. In contrast, if host-derived cells are the APC,

Table II. *Efficiency of chimera formation and T cell depletion in A/J→F_1 and B6→F_1 bone marrow chimeric mice*[a]

Expt. No.	Chimera	T Cell Depletion	% Cells		% Donor Genotype	
			$CD4^+$	$CD8^+$	A/J	C57BL/6
1	A/J→F_1	None	ND	ND	100	
2	A/J→F_1	None	ND	ND	100	
3	A/J→F_1	Undepleted	19.0	9.2		
		CD8	33.9	0.6	99.7	
		CD4	0.2	6.3	99.5	
4	B6→F_1	None	ND	ND	ND	ND
5	B6→F_1	Undepleted	17.4	12.2		
		CD8	32.1	0.1		97.9
		CD4	1.2	13.9		100

[a] To determine the efficiency of bone marrow reconstitution, splenocytes of bone marrow chimeric mice were tested by indirect immunofluorescence with donor and recipient genotype MHC class I-specific mAbs (16-3-1 for H-$2K^k$; 28-13-3 for H-$2D^b$). To determine efficiency of T cell depletion, splenocytes of $CD4^+$ or $CD8^+$ T cell depleted mice were tested by indirect immunofluorescence for CD4 and CD8 expression (GK1.5, 2.43 mAbs, respectively).

then the chimeras will have HEL-specific T cells restricted to the genotype of the host (A) regardless of the genotype of the tumor. To test these alternatives, A/J→ F_1 and B6→F_1 chimeras were generated and challenged i.p. with SaI/A^b/HEL or SaI/A^k/HEL tumor cells, respectively. Seven days after tumor cell immunization, spleens were removed and depleted of B cells and adherent cells by panning, and the remaining T cells were incubated in vitro with lysozyme plus fresh A/J or C57BL/6 splenocytes as APC. Supernatants were harvested 24 h later and tested for IL-2 content. Since the goal of these experiments is to characterize the earliest Ag-specific response, T cells were tested 1 wk postimmunization. Likewise, T cells were assayed for IL-2 secretion without an in vitro or in vivo boost to the Ag (HEL), so that the primary or initial response would be measured.

A/J→F_1 and B6→F_1 bone marrow chimeras were generated as described in *Materials and Methods*. The efficiency of chimera formation was determined using indirect immunofluorescence to measure the percentage of donor genotype cells in the recipients' spleens. Table II lists the chimeras used in subsequent experiments and shows the MHC class I genotype (*H-2K*^{k} for A/J vs *H-2D*^{b} for C57BL/6) of splenocytes from the chimeras as measured by immunofluorescence. As shown, all the chimeras used in the following experiments were ≥97.9%, and most chimeras were ≥99.5%, of the donor genotype. Chimeras were also tested functionally for hemopoietic reconstitution. Splenocytes of representative chimeras were used as APC for intact HEL to I-A^k- and I-A^b-restricted HEL-specific hybridomas, 3A9 and JK1290, respectively. A/J→F_1 and A/J splenocytes presented HEL to 3A9 hybridoma cells, but not to JK1290 hybridoma cells. In contrast, B6→F_1 and C57BL/6 splenocytes presented Ag to JK1290 cells, but not to 3A9 hybridoma cells (data not shown). APC of the bone marrow chimeras, therefore, are phenotypically and functionally the donor genotype.

Table III shows the results of three representative, independent experiments assessing IL-2 production by total T cells from tumor-immunized, chimeric mice. In two experiments (Expt. 1 and 2) A/J→F_1 chimeras were immunized with SaI/A^b/HEL tumor cells, while in one experiment (Expt. 4), B6→F_1 chimeras were immunized with SaI/A^k/HEL tumor cells. In all three experiments HEL-specific T cells restricted to both the tumor and the host genotype were present. IL-2 release by immune T cells cocultured in vitro with lysozyme plus irrelevant genotype APC (SWR, *H-2*^{q}) was at background levels (data not shown). Therefore, at 1 wk postimmunization with genetically modified tumor cells, both tumor cells

Table III. *Both tumor cells and host-derived APC present tumor-encoded Ag to CD4$^+$ T cells in A/J→F$_1$ and B6→F$_1$ bone marrow chimeric mice*[a]

Expt. No.	Tumor Cells	Chimera	T Cell Depletion	T Cell: APC Ratio	IL-2 (pg/ml) A/J APC	IL-2 (pg/ml) C57BL/6 APC
1	Sal/A^b/HEL	A/J→F$_1$	—	10:1	55	357
				50:1	607	520
2	Sal/A^b/HEL	A/J→F$_1$	—	10:1	430	172
3	Sal/A^b/HEL	A/J→F$_1$	CD8	10:1	217	137
			CD4	10:1	7.3	4
4	Sal/A^k/HEL	B6→F$_1$	—	10:1	132	33
5	Sal/A^k/HEL	B6→F$_1$	CD8	10:1	0	197
			CD4	10:1	0	10.6

[a] A/J→F$_1$ or B6→F$_1$ bone marrow chimeric mice were challenged i.p. with Sal/A^b/HEL or Sal/A^k/HEL tumor cells, respectively, and splenic T cells were tested in vitro for IL-2 production in response to lysozyme plus I-A^b (C57BL/6) or I-A^k (A/J) APC, respectively. In some experiments, responding T cells were depleted for CD4$^+$ or CD8$^+$ T cells prior to assay.

and host bone marrow-derived cells were APC for tumor-encoded Ags.

The genetically modified tumor cells constitutively express MHC class I (H-2K^k, H-2D^d, and H-2L^d) as well as the transfected MHC class II (I-A^k or I-A^b) molecules. Since CD4$^+$ and CD8$^+$ T cells are activated by Ag in the context of MHC class II and class I molecules, respectively, and since both T cell types can synthesize IL-2, it is possible that the read-out of the splenic T cell experiments reflects HEL-specific, class I-restricted CD8$^+$ T cells. To identify the responding splenic T cells, chimeric mice were depleted of CD4$^+$ or CD8$^+$ T cells before and during immunization, and the remaining T cells were tested for IL-2 secretion following HEL presentation in vitro. Two representative experiments are shown in Table III, one experiment using A/J→F$_1$ chimeric mice immunized with Sal/A^b/HEL tumor cells (Expt. 3) and a second experiment using B6→F$_1$ chimeric mice immunized with Sal/A^k/HEL tumor cells (Expt. 5). As shown in Table II, mice depleted for CD4$^+$ or CD8$^+$ T cells had ≤1.2 or 0.6% of these cells, respectively, demonstrating functional depletion of these populations. As shown in Table III, in both the depletion experiments most of the IL-2 activity was produced by CD4$^+$ T cells, since depletion of CD4$^+$ T cells reduced IL-2 activity to negligible levels. Consistent with the results of Expt. 1, 2, and 4, in Expt. 3, both tumor genotype and host genotype APC stimulated HEL-specific IL-2 release, indicating that both tumor cells and host-derived cells are APC for tumor-encoded Ag (HEL). In contrast, in Expt. 5, only host genotype (*H-2^b*) cells stimulated IL-2 secretion, suggesting that in these mice only host-derived cells are the APC for tumor-encoded Ags.

The five experiments shown in Table III are representative of 25 similar experiments performed using chimeric or (C57BL/6 × A/J)F$_1$ mice. Results similar to those of Expt. 5, in which only host genotype APC stimulated IL-2 secretion, were noted in only two of these experiments, while the remaining experiments all showed either host and tumor genotype presentation, or tumor genotype presentation alone. It was occasionally observed that tumor-encoded Ags were exclusively presented by host-derived cells; however, such presentation was a relatively rare event. Similar experiments using class II$^-$ Sal/HEL tumor cells showed no direct Ag presentation (data not shown), indicating that direct Ag presentation by tumor cells to CD4$^+$ T cells requires MHC class II expression by the tumor. Therefore, as measured at 1 wk postimmunization, CD4$^+$ T cells specific for tumor-encoded Ags were activated via Ag presentation by both tumor and host-derived cells, indicating that both direct and indirect (cross-priming) pathways are used.

Discussion

During an in vivo immune response, CD4$^+$ T lymphocytes are activated by an Ag-specific signal plus a second or costimulatory signal (29). The Ag-specific signal consists of antigenic peptide bound to a MHC class II molecule, which interacts with the corresponding TCR and CD4 complex on the responding T cell. The second signal consists of a costimulatory molecule, such as B7, which binds to its cognate receptor, CD28, on the responding T cell. Since only certain cells, such as dendritic cells, macrophages, activated B lymphocytes, or Langerhans cells, express MHC class II and either constitutively express or are inducible for costimulatory molecules (29), only these so-called professional APC are thought to activate CD4$^+$ T lymphocytes. In our recently developed immunotherapeutic strategy, tumor cells are transfected with genes encoding syngeneic MHC class II molecules or MHC class II plus B7 molecules (4, 7). The class II transfectants are excellent immunogens for vaccinating tumor-free mice against subsequent challenge with wild-type tumor, and during the immunization process the transfectants are induced to express B7-1 and B7-2 molecules (30). Although the single transfectants (MHC class II) are not effective immunogens in tumor-bearing mice, double transfectants (MHC class II plus B7) are potent immunotherapeutic agents for the treatment of established solid tumors (12) and metastatic disease[4] (31). We have hypothesized that such genetically modified tumor cells are APC for tumor-encoded Ags because they deliver both the Ag-specific signal and the costimulatory signal to CD4$^+$ T cells. The studies reported here confirm this hypothesis by demonstrating that during the immunization process, class II-transfected tumor cells are APC for endogenously encoded tumor molecules. Our immunotherapeutic approach, therefore, provides an alternative pathway for activation of CD4$^+$ T lymphocytes. Presumably, the ability of the class II transfected tumor cells to function as APC and directly activate CD4$^+$ T cells is responsible for their potent immunotherapeutic effect.

Our data indicate that both tumor cells and host-derived cells are APC for tumor-encoded HEL. These experiments were designed to test Ag presentation of an intracellular, surrogate tumor Ag; however, ELISA assays of Sal/A^k/HEL supernatants show low levels of secreted HEL. Since the Sal/A^k/HEL transfectants are "leaky" for HEL, one cannot determine whether intracellular tumor Ag presentation is normally indirect via host-derived APC, or if cross-priming occurs because soluble HEL is available. If leakiness is the reason for the observed cross-priming, then the relative roles of direct vs indirect presentation for bonafide tumor Ags will be dependent on the cellular locale and behavior of tumor Ag.

Cross-priming or indirect Ag presentation is the usual route for activation of CD4$^+$ T cells. In this process, tumor Ag (in this case, lysozyme) is released from tumor cells, trafficks via the lymphatic and/or circulatory system to the closest regional lymph node(s), and is internalized by professional APC within the lymph node. Alternatively, Ag may be internalized in the periphery by professional APC and brought to the regional lymph node by the APC. In either case, internalized Ag is processed by the APC and presented as peptide in the context of MHC class II molecules on the surface of the APC (32). Since only a small percentage of CD4$^+$ T cells has the appropriate receptor for a given Ag, localization of Ag to the regional lymph node allows exposure to the maximal

[4] B. Pulaski and S. Ostrand-Rosenberg. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with MHC class II and B7.1 cell-based tumor vaccines. *Submitted for publication.*

number of $CD4^+$ T cells. Trafficking of Ag to the regional lymph node during a conventional immune response, therefore, optimizes contact of Ag with the appropriate T cells.

If tumor cells are the APC, Ag presentation could occur either at the tumor site or in the regional lymph node. Presentation at the tumor site is unlikely, since unless a very large number of $CD4^+$ T cells traffick to the site, it is improbable that T cells with an appropriate TCR will be exposed to Ag. Trafficking of the transfectants to the regional nodes is more likely. Tumors are known to metastasize via the lymphatic circulation, and a recent study using genetically modified, nonmetastatic tumor cells demonstrated tumor cells in draining nodes (33). The class II-transfected tumor cells, therefore, may migrate to the draining lymph nodes; however, the precise logistics of the Ag presentation process remain unclear.

Although the activation of $CD4^+$ T cells by genetically modified tumor cells has not been previously studied, other investigators have examined the activation of $CD8^+$ T cells during immunization with transfected tumor cells. Three studies have examined Ag presentation by cytokine modified tumor cells (granulocyte-macrophage CSF, IL-4, IL-7, or IL-3) and have found that cytokine expression facilitates cross-priming (34, 35) or that tumor-specific $CD8^+$ T cells are exclusively activated by host-derived APC (36). Two of these studies also investigated whether B7-transfected tumor cells directly or indirectly presented Ag to tumor-specific $CD8^+$ T cells. In one study, Ag presentation was exclusively via cross-priming (37), while in the other report, Ag presentation was directly via the B7-transfected tumor cells (34). Most of these results, therefore, were unexpected, since cytokine and B7-transfected tumor cells were presumed to induce tumor-specific immunity by direct presentation of tumor Ags to T cells (1, 3, 5).

There are several potential explanations for the observed differences between previously reported studies and the results of the present report. 1) The present report addresses Ag presentation to $CD4^+$ T cells, while earlier studies examined presentation to $CD8^+$ T cells. The varying results could be due to differences in Ag presentation to $CD4^+$ vs $CD8^+$ T cells. 2) In this report, Ag presentation during the primary response (7 days postpriming) was studied, while earlier reports examined later time points (35) and/or secondary responses (34, 36, 37). Since tumor cells remain intact in vivo for only a limited time, direct Ag presentation may occur during the early stage of the immunization process while tumor cells remain intact, and indirect Ag presentation may dominate during later stages when tumor debris is available. 3) In two of these studies (36, 37), the transfected tumor cells were irradiated, and secretion of the surrogate tumor Ag was not assessed. If soluble Ag was available, cross-priming would be the expected result. 4) One of these studies (35) was not designed to measure direct Ag presentation, so even if direct presentation by genetically modified tumor cells occurred, it would not have been detected.

In contrast, studies by Kündig and colleagues (38) demonstrated that fibroblasts transfected with a viral Ag, if injected into a lymph node, directly present Ag to $CD8^+$ T cells, thereby stimulating potent viral-specific $CD8^+$ CTL. The logistics of $CD8^+$ T cell activation by genetically modified therapeutic cells are, therefore, unclear, with data supporting both direct and indirect pathways.

The finding that genetically modified tumor cells directly activate $CD4^+$ T cells has clear implications for the design of immunizing and immunotherapeutic agents. If cell-based immunogens are to be considered as vaccines and/or immunotherapeutic agents, then the cells should be engineered to optimally present Ag to $CD4^+$ T cells. If Ag presentation activity correlates with immunization potential, measurements of in vitro Ag presentation activity may be prognostic of therapeutic efficacy. Depending on the desired type of Th cell (Th1 vs Th2), immunizing cells could be tailored to facilitate activation of a particular subpopulation, perhaps via coexpression or simultaneous bolus administration of cytokines favoring differentiation of one or the other helper population (i.e., IL-12, IL-10, etc.).

Although concern has been voiced about using tumor cell-based immunogens in a clinical setting, a significant number of phase I or II clinical trials using tumor cell material have been completed or are in progress (http://cancernet.nci.nih.gov). Since these trials have not identified any significant safety issues and in some cases have shown modest therapeutic responses (despite overwhelming tumor load), the clinical use of tumor cell-based immunogens is feasible. All the trials using cell-based strategies have been exclusively aimed at enhancing $CD8^+$ T cell responses. The data presented in this report combined with published therapeutic studies (12) strongly argue that an optimal cell-based vaccine should also target the activation of $CD4^+$ T lymphocytes.

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Gene Therapy

Class II-Transfected Tumor Cells Directly Present Endogenous Antigen to $CD4^+$ T Cells In Vitro and Are APCs for Tumor-Encoded Antigens In Vivo

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Summary: We have previously demonstrated that class II-transfected tumor cells are very effective immunogens that protect against wild-type primary and metastatic tumor and, if supertransfected with genes encoding co-stimulatory molecules, are immunotherapeutic agents that successfully treat mice with established solid tumor. These results are consistent with our hypothesis that the class II-transfected tumor cells act as antigen-presenting cells (APCs) that directly activate tumor-specific $CD4^+$ T cells; however, direct data supporting this hypothesis are lacking. In the present study, we test this hypothesis using class II-transfected tumor cells supertransfected with the hen egg lysozyme gene as a surrogate tumor antigen. In vitro antigen presentation assays demonstrate that class II-transfected tumor cells present to $CD4^+$ T cells endogenously encoded tumor antigen, provided they do not co-express the class II-associated invariant chain. In vivo experiments using genetically marked tumor cells and host APCs demonstrate that both class II-transfected tumor cells and host cells are APCs for tumor-encoded antigens, although tumor cells appear to dominate the response. These results support the hypothesis that the immunogenicity and therapeutic value of class II-transfected tumor cells stem from their ability to function as APCs for tumor-encoded antigens and directly activate tumor-specific $CD4^+$ T lymphocytes. **Key Words:** Major histocompatibility complex class II—Tumor immunity—Antigen presentation—$CD4^+$ T cells.

Although some malignancies can be cured by conventional therapies, many metastatic cancers and some primary tumors are not amenable to surgery and are refractile to radiation and/or chemotherapy. In these situations, an optimized antitumor immune response could play a valuable role in specifically limiting or eliminating tumor cell proliferation. Since metastatic disease and recurrence of primary tumor may occur over a relatively prolonged period, effective immunity would optimally include long-term immunological memory. Many of the novel anticancer immunotherapeutic strategies that are currently under development focus on the activation of $CD8^+$ cytotoxic T cells. Although $CD8^+$ T cells are very effective agents for inhibiting tumor growth, their optimal activation is dependent on the co-activation of $CD4^+$ T helper cells (1,2). Likewise, maximal long-term immunological memory may be facilitated by the activation of $CD4^+$ T helper lymphocytes. Since our goal is to achieve maximum activation of tumor-specific $CD8^+$ T

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cells and long-term immunity, we have focused on the activation of tumor-specific $CD4^+$ T cells.

Our strategy for activating tumor-specific $CD4^+$ T cells has been to transfect tumor cells with syngeneic major histocompatibility complex (MHC) class II genes and use the genetically modified tumor cells as a cell-based vaccine. This strategy is based on the premise that class II-expressing tumor cells may be effective immunogens because they directly present tumor-encoded antigens to $CD4^+$ T helper lymphocytes and thereby induce T cell-mediated immunity and long-term immunological memory.

In our previous studies, MHC class II-transfected tumor cells have been shown to be very effective immunogens for protection against wild-type primary and metastatic tumor (3,4) and, when additionally transfected with genes encoding co-stimulatory molecules, are therapeutic agents for the treatment of mice with established solid tumor (5). The therapeutic efficacy of class II transfectants is consistent with our hypothesis that the transfectants are antigen-presenting cells (APCs); however, direct data supporting this hypothesis are lacking. Since a better understanding of the mechanism by which the transfectants induce tumor immunity may lead to improved therapy, the present studies were undertaken to determine if class II-transfected tumor cells act as APCs during the immunization process.

MATERIALS AND METHODS

Cells

SaI tumor cells are an A/J-derived ($K^kA^kE^kD^d$) mouse sarcoma that has been previously described (6). SaI transfectants expressing I-A^k (6), I-A^b, and/or hen egg lysozyme (HEL) (33) were generated as previously described. Class II and lysozyme transfectants were maintained in medium supplemented with 400 μg/ml G418 and/or hygromycin, respectively. Human melanoma cell lines mel 1011, mel 1102, mel 1088, Jxmel, and 14932 and human breast cancer cell lines MCF-7, SKBR-3, and MDA-435 were generously provided by Drs. M. Nishimura, M. Brady, and G. Parmiani. The human tumor lines were maintained in Isacove's modified Dulbecco's medium supplemented with 15% fetal calf serum, 1% penicillin, 1% streptomycin, 1% gentamicin, and 1% Glutamax.

Antibodies

The hybridoma secreting the pan HLA-DR monoclonal antibody (mAb) L243 was obtained from ATCC. The hybridoma secreting the PIN1.1 mAb to the human invariant chain was kindly provided by Dr. M. Marks. Both mAbs were purified on Protein G as previously described (6). A rabbit polyclonal antiserum to HLA-DM was generously provided by Dr. D. Zaller. GK1.5 (CD4) and 19/178 (CD8) were used for in vivo depletion studies as previously described (7).

Bone Marrow Chimeras

Bone marrow chimeras were generated as previously described (8) with minor modifications. (C57BL/6 × A/J) F1 female mice to be reconstituted were maintained on tetracycline drinking water (0.2%) for 1 week prior to and 5 weeks after reconstitution. On the day of bone marrow transfer, recipients were lethally irradiated (900 rads total body, ^{137}Cs source) and reconstituted intravenously with donor C57BL/6 or A/J female marrow at a ratio of 1:2 donor/recipient. Reconstituted mice were given a 7-day course of subcutaneous gentamicin sulfate (0.5 mg/mouse) beginning 1 day before reconstitution. Chimeras were used between 6 and 12 weeks postreconstitution and were monitored for reconstitution efficiency by immunofluorescence analysis of the MHC class I genotype of their spleens. Splenocytes of all chimeras used in these experiments were 100% of the donor bone marrow MHC genotype.

Immunofluorescence

Tumor cells and splenocytes were stained by indirect immunofluorescence and analyzed on an Epics XL flow cytometer as previously described (3). Percent positive cells for the human melanoma and breast cancer cell lines was calculated using the Epics Immuno-4 program as follows. Control fluorescent conjugate staining was subtracted from specific antibody staining for each cell line to yield percent positive cells. To normalize for "nonspecific" staining of known negative cells, the percent positive cells for a negative control cell line (usually Jurkat cells) was then subtracted from the percent positive cells for each experimental tumor. Resulting values were pooled for multiple experiments and are presented as percent positive cells ± SE.

Antigen Presentation Assays

Two types of APC assays were performed. In the first type, genetically modified, irradiated tumor cells (SaI, SaI/A^k, SaI/A^k/Ii or their lysozyme-transfected counterparts; 5×10^4 cells/100 μl) served as the APCs and were co-cultured overnight with the I-A^k-restricted HEL

46-61-specific T cell hybridoma, 3A9 (5×10^4 cells/100 μl). In the second type of APC assay, chimeric mice were immunized intraperitoneally with the various lysozyme-transfected tumor cells and their spleens removed 1 week later and depleted for B lymphocytes by panning (9). These responder cells were cultured overnight with naive splenocytes (APCs) and intact lysozyme. For both types of APC assays, interleukin-2 (IL-2) content of the supernatants was determined by enzyme-linked immunosorbent assay (ELISA) using Endogen kits as per the manufacturer's instructions. IL-2 values are expressed as picograms per milliliter.

RESULTS

Class II-Transfected Sarcoma Cells Present Endogenously Synthesized Antigen In Vitro

To determine if class II-transfected tumor cells directly present antigen to $CD4^+$ T cells, we have generated tumor cells expressing an endogenously encoded antigen and tested the transfectants for their ability to present this antigen to an I-A^k-restricted T cell hybridoma. We have chosen the antigen HEL for these studies for two reasons: (a) The SaI sarcoma tumor antigen is unknown, while lysozyme has a well characterized immune response and hence is an appropriate surrogate endogenous tumor antigen. (b) Different tumor antigens localize to various intracellular compartments, and by using gene constructs with different trafficking signals, we can generate transfectants that express lysozyme in these various intracellular locales. Wild-type SaI and class II-transfected (SaI/A^k) sarcoma tumor cells were therefore supertransfected with HEL constructs containing either an endoplasmic reticulum (ER) retention signal [erHEL (10)] or the HEL gene fused to the transmembrane region of an H-$2K^b$ MHC class I gene [mHEL (11)]. The resulting transfectants, SaI/A^k/erHEL and SaI/A^k/mHEL, express lysozyme in the ER and the plasma membrane, respectively, as measured by indirect immunofluorescence (data not shown).

Table 1 shows the results of three antigen presentation experiments in which the lysozyme transfectants and wild-type (lysozyme-negative) SaI sarcoma cells were co-cultured with the I-A^k-restricted, HEL-specific T cell hybridoma 3A9, and IL-2 levels measured by ELISA. In all experiments, the class II-transfected tumor cells expressing either erHEL or mHEL stimulated IL-2 release, while tumor cells not expressing class II or HEL did not.

Class II^+ professional APCs usually present exogenously synthesized rather than endogenously synthesized antigen (12). The preferential presentation of exogenous antigen is at least in part due to co-expression of the class II-associated invariant (Ii) chain, which is thought to inhibit the binding of endogenous antigen to the class II heterodimer in the ER (13). Since our previous studies (14) have demonstrated that Ii^+ class II-transfected tumor cells are not immunogenic, we would predict that class II^+ Ii^+ SaI cells (SaI/A^k/Ii) would not function as APCs for endogenously synthesized lysozyme. To test this hypothesis, SaI/A^k/Ii cells were transfected with either the erHEL or the mHEL constructs to generate SaI/A^k/Ii/erHEL or SaI/A^k/Ii/mHEL cells, respectively. The transfectants were tested by indirect immunofluorescence and shown to express approximately equivalent levels of lysozyme (data not shown). As shown in Table 1, neither of these transfectants presented lysozyme. Class II-transfected sarcoma cells therefore present endogenously synthesized antigen provided they do not co-express Ii. Since previous studies demonstrated that SaI/A^k but not SaI/A^k/Ii cells are effective immunogens against wild-type tumor (14), there appears to be a strong correlation between the ability to present endogenous antigen and immunotherapeutic efficacy.

TABLE 1. *Class II-transfected sarcoma cells present endogenously synthesized antigen*

APCs	IL-2 (pg/ml) Expt. 1	Expt. 2	Expt. 3
SaI	0	0	0
SaI/A^k	0	13.2	0
SaI/erHEL	0		0
SaI/A^k/erHEL	79.2		125.4
SaI/A^k/Ii/erHEL-7	0		
SaI/A^k/Ii/erHEL-9	0		
SaI/mHEL		0	0
SaI/A^k/mHEL		316.8	105.6
SaI/A^k/Ii/mHEL-6		0	
SaI/A^k/Ii/mHEL-9		0	

APCs were mixed with the I-A^k-restricted, lysozyme-specific T cell hybridoma 3A9 at ratios of 5×10^4 or 10^5 APCs to hybridoma and cultured for 24 h. Supernatants were removed and assayed for IL-2 activity by ELISA. See text for abbreviations.

Human Melanoma and Breast Cancer Tumor Cells That Express HLA-DR Co-Express Invariant Chain

A number of immunotherapeutic protocols propose to use autologous tumor cells as immunogens. The concept behind these studies is that the tumor cells will present endogenously synthesized tumor peptides and therefore immunize against wild-type tumor. Since the data of Table 1 indicate that only those tumor cells expressing MHC class II without co-expression of Ii will be effective immunogens for $CD4^+$ T cells, we have surveyed a

variety of human tumor cells to determine if class II expression is independent of Ii expression. Five human melanoma (mel 1011, mel 1102, mel 1088, 14932, and Jxmel) and four human breast carcinoma (MCF-7, SKBR-3, MDA-431, and MDA-435) lines were tested by indirect immunofluorescence for plasma membrane HLA-DR expression using the pan-DR mAb L243, for internal Ii expression using the PIN1 mAb, and for internal HLA-DM expression using a rabbit polyclonal antiserum to recombinant HLA-DM. As shown in Table 2, different human tumor lines express varying percentages of HLA-DR-positive cells. Although for each tumor line the percent of Ii^+ and HLA-DM^+ cells is generally lower than the percent of HLA-DR^+ cells, there is a rough linear correlation between HLA-DR, Ii, and HLA-DM expression within each cell line. Positive control B cells (JB) show a similar linear relationship between HLA-DR, Ii, and HLA-DM expression, and T cells (Jurkat) are essentially negative for these molecules. Human tumor cells that constitutively express HLA-DR therefore co-express Ii and HLA-DM. If the results of the mouse studies of Table 1 extend to human tumor cells, then human tumor cells that constitutively express MHC class II molecules are unlikely to be effective immunogens for endogenously synthesized tumor antigens because of their co-expression of Ii chain.

During In Vivo Immunization Process in (C57BL/6 × A/J)F1 Mice, Both Tumor Cells and Host-Derived Cells Are APCs for Tumor-Encoded Antigens

The results of Table 1 demonstrate that class II-transfected tumor cells can present endogenous antigen; however, they do not determine if the tumor cells are the functional APCs in vivo during the immunization process. To identify the functional APCs for $CD4^+$ T cells, we have modified a genetic approach first developed by Bevan and colleagues (15,16). In this experiment, er-HEL-transfected SaI tumor cells expressing an MHC class II of one genotype (SaI/A^k/erHEL or SaI/A^b/erHEL) are inoculated into semisyngeneic (C57BL/6 × A/J)F1 mice of the H-2^a/H-2^b genotype. (Note: H-2^a is a recombinant genotype consisting of H-$2K^kA^kD^d$.) If the tumor cells are the exclusive APCs, then HEL-specific T cells restricted to the MHC class II genotype of the inoculated tumor cells will be produced. However, if host-derived APCs and tumor cells, or if only host-derived cells, are the functional APCs, then HEL-specific T cells restricted to both the H-2^a and the H-2^b genotypes will be produced. (C57BL/6 × A/J)F1 mice were challenged intraperitoneally with SaI/A^k/erHEL or SaI/A^b/erHEL tumor cells. One week later, spleens were harvested, B cells removed by panning, and the resulting T cells incubated with fresh A/J (H-2^a) or C57BL/6 (B6, H-2^b) splenic APCs pulsed with 1 mg/ml lysozyme. Supernatants were removed after 24 h and analyzed for IL-2 content by ELISA. Since IL-2 is produced by both $CD4^+$ and $CD8^+$ T cells, in some experiments, F1 mice were depleted of $CD4^+$ or $CD8^+$ T cells prior to and during immunization by in vivo mAb treatment so that IL-2 production by specific T cell subpopulations could be assessed.

TABLE 2. *Human melanoma and breast cancer lines that express HLA-DR co-express invariant chain*

		% positive cells ± SE		
Tumor	Cell line	HLA-DR	Ii	DM
Melanoma	mel 1011	13 ± 3	0	14 ± 3
	mel 1088	69 ± 14	33 ± 10	6
	mel 1102	39 ± 7	24 ± 7	0
	Jxmel	88 ± 2	65	
	14932	58 ± 5	32	
Breast carcinoma	MCF-7	38 ± 9	33 ± 11	28 ± 20
	SKBR-3	17 ± 1	39	22
	MDA-435	48 ± 13	55 ± 5	49 ± 10
	MDA-231	40 ± 5	25 ± 12	9 ± 5
B cell	JB	66 ± 9	50 ± 17	30 ± 4
T cell	Jurkat	0	1.5	34

Cells were stained for HLA-DR (L243 mAb), Ii (PIN1.1 mAb), or HLA-DM (rabbit anti-human polyclonal antibody) and secondarily stained with the appropriate fluorescent conjugate. See Materials and Methods for the calculation determining % positive cells for each sample. mAb, monoclonal antibody.

Several different results were obtained. An experiment representative of each type of result is shown in Table 3. In experiment 1, SaI/A^k/erHEL cells were the immunizing tumor cells, and only H-2^a-restricted, HEL-specific T cells were induced, indicating that only the tumor cells present antigen. However, in experiment 2, in which SaI/A^b/erHEL cells were the immunizing tumor cells, both H-2^a and H-2^b-restricted, HEL-specific T cells were induced, suggesting that either host-derived cells or host-derived cells plus tumor cells are the relevant APCs. To determine if the T cells are class I and/or class II restricted (i.e., $CD4^+$ and/or $CD8^+$), the F1 mice were depleted of $CD4^+$ or $CD8^+$ T cells prior to and during the immunization. As shown in Table 3, in experiments 3 and 4, the SaI/A^k/erHEL tumor cells induce HEL-specific $CD4^+$ T cells restricted to both genotypes, while the SaI/A^b/erHEL cells induce HEL-specific $CD4^+$ and $CD8^+$ T cells restricted to both genotypes. The SaI/A^k/erHEL tumor cells therefore predominantly induce I-A^k-restricted, $CD4^+$ HEL-specific T cells, suggesting that the tumor cells are the dominant APCs. In contrast, the SaI/A^b/erHEL tumor cells induce approximately equivalent levels of both I-A^k- and I-A^b-restricted $CD4^+$ T cells,

TABLE 3. *In (A/J × B6)F1 mice, both class II-transfected tumor cells and host-derived cells are APCs for tumor-encoded antigens*

			IL-2 (pg/ml)			
			A/J APC		B6 APC	
Experiment	Tumor	Treatment	10:1	50:1	10:1	50:1
1	SaI/A^k/HEL	—	370.9	295	0	0
2	SaI/A^b/HEL	—	821.7		918.1	
3	SaI/A^k/HEL	—	533.9		0	
		CD8 depleted	529.9		296.3	
		CD4 depleted	139.9		0	
4	SaI/A^b/HEL	—	505.6		420.4	
		CD8 depleted	147.8		483.8	549.8
		CD4 depleted	254.8		215.2	

(C57BL/6 × A/J)F1 mice were challenged intraperitoneally with 10^6 SaI/A^k/HEL or SaI/A^b/HEL tumor cells. One week later spleens were removed and splenocytes assayed in vitro for IL-2 production in response to A/J or C57BL/6 APCs pulsed with lysozyme. Responder/stimulator ratios in the APC assay were either 10:1 or 50:1. See text for abbreviations.

suggesting that both host-derived cells and tumor cells can function as APCs for tumor-encoded antigen. The SaI/A^k/erHEL immunization experiments therefore suggest that the tumor cell is the functional APC; however, the SaI/A^b/erHEL experiments do not distinguish whether tumor cells or host cells are the relevant APCs.

During In Vivo Immunization Process in Parent → F1 Chimeras, Both Tumor Cells and Host-Derived Cells Are APCs for Tumor-Encoded Antigens

Since the experiments of Table 3 using (C57BL/6 × A/J)F1 mice do not clearly distinguish which cells are the functional APCs during immunization, we have performed additional experiments to identify the relevant APCs. As in the experiments of Table 3, we have used (C57BL/6 × A/J)F1 mice; however, for these experiments the F1 mice were lethally irradiated and reconstituted with either C57BL/6 or A/J bone marrow so that the resulting chimeric mice contained either H-2^b or H-2^a hematopoietic cells, respectively (B6 → F1 or A/J → F1 chimeras). The chimeras were then challenged with the appropriate tumor cells such that the MHC class II genotype of the tumor differed from that of the chimera (i.e., B6 → F1 chimeras were immunized with SaI/A^k/erHEL tumor cells, while A/J → F1 chimeras were immunized with SaI/A^b/erHEL.) If the tumor cells are the only APCs for tumor antigens, then the resulting HEL-specific T cells will be restricted exclusively to the tumor genotype. In contrast, if host-derived cells are the APCs for tumor antigens, then the resulting HEL-specific T cells will be exclusively restricted to the host MHC genotype. If both the tumor cells and the host cells are APCs, then the HEL-specific T cells will be restricted to both MHC genotypes.

As in the experiments of Table 3, bone marrow chimeras were challenged intraperitoneally with SaI/A^k/erHEL or SaI/A^b/erHEL tumor cells, and the spleens were removed 7 days later and co-cultured with C57BL/6 or A/J APCs pulsed with lysozyme. Supernatants from the cultures were then tested for IL-2 content by ELISA. As shown in Table 4, if total T cells are assessed for IL-2 production (experiments 1 and 2), immunization with SaI/A^b/erHEL or SaI/A^k/erHEL tumor

TABLE 4. *In bone marrow chimeras, both tumor cells and host cells are APCs for tumor-encoded antigens*

				IL-2 (pg/ml)	
Experiment	Bone marrow chimera	Tumor cells	Treatment	A/J APC	B6 APC
1	A/J→F1	SaI/A^b/HEL	—	55	357
2	B6 →F1	SaI/A^k/HEL	—	132	33
3	A/J→F1	SaI/A^b/HEL	CD8 depleted	217	137
			CD4 depleted	7.3	4

(C57BL/6 × A/J)F1 mice were lethally irradiated and reconstituted with A/J (A/J → F1) or C57BL/6 (B6 → F1) bone marrow. Five to six weeks after reconstitution, chimeras were challenged with SaI/A^b/HEL or SaI/A^k/HEL tumor cells, respectively. One week after tumor challenge, spleens were removed and assayed in vitro for IL-2 production in response to A/J or C57BL/6 APC pulsed with lysozyme. Responder/stimulator ratio was 10:1. See text for abbreviations.

cells results in preferential presentation by the tumor genotype, H-2^b and H-2^a, respectively, indicating that the tumor cells are the predominant APCs, although host-derived cells minimally present antigen. To identify which T cells are activated during the immunization procedure, mice were depleted of $CD4^+$ or $CD8^+$ T cells prior to and during the immunization by treatment with mAbs to $CD4^+$ or $CD8^+$. As shown in Table 4, depletion of $CD4^+$ T cells results in very low IL-2 production in response to either genotype, while depletion of $CD8^+$ T cells leaves significant IL-2 production in response to both tumor and host genotype. The bone marrow chimera experiments therefore indicate that at 1 week after immunization, tumor-specific $CD4^+$ cells are the predominantly activated class of T cells and that although both tumor and host-derived cells function as APCs, tumor cells are the predominant APCs.

DISCUSSION

As novel antitumor immunotherapeutic approaches are developed, it is imperative to define the mechanism of antigen-specific T cell activation so that the therapies can be further improved. We have therefore directly tested whether class II-transfected tumor cells can function as APCs for tumor-encoded antigens and if the transfectants actually present tumor-encoded antigen in vivo during the immunization process. The in vitro APC experiments of Table 1 and in vivo immunization experiments of Tables 3 and 4 directly demonstrate that class II-transfected tumor cells are capable of presenting tumor-encoded peptides to $CD4^+$ T cells and that during the immunization process, the tumor cells are the functional APCs for tumor-encoded antigens. Since our previous experiments have shown that SaI/A^k cells are highly immunogenic and rejected by autologous mice, while SaI/A^k/Ii tumor cells are poorly immunogenic and lethal (14), there is also a strong correlation between the ability of tumor cells to present endogenous antigen and their immunogenicity. Therefore, it is very likely that the potent antitumor immunity induced by the class II transfectants is due to their ability to function as APCs for tumor peptides and thereby directly activate tumor-specific $CD4^+$ T lymphocytes.

Although the class II-transfected tumor cells present antigen, they are not as efficient APCs as professional APCs. Their decreased efficiency may be because they do not optimally process and/or present tumor-encoded antigens. Both processes are complex phenomena and require numerous accessory molecules and organelles for optimal function (17,18). Since APC activity is strongly correlated with induction of tumor-specific immunity, enhanced immunogenicity may result if tumor cells are made even more efficient APCs for tumor antigens. Indeed, tumor cell expression of co-stimulatory molecules, which are known to facilitate T cell activation (19), has been shown in several tumor systems to amplify tumor cell immunogenicity (5,7,20,21). Identification of additional genes facilitating antigen presentation to $CD4^+$ T cells and their expression in tumor cells may therefore further improve the vaccine potential of tumor cells.

The ability of genetically modified tumor cells to present tumor-encoded antigen is clearly reduced if the tumor cells co-express the class II-associated Ii protein. Since class II and at least some of its accessory proteins (Ii and HLA-DM) are coordinately transcriptionally regulated (22), most tumor cells that constitutively express MHC class II molecules would be expected to co-express Ii and HLA-DM. Our survey of human melanoma and breast cancer cell lines (Table 2) corroborates this co-expression, although the ratio of class II, Ii, and DM molecules in the various tumor lines tested fluctuates greatly. The absence of effective immunity to tumor cells that constitutively express MHC class II molecules therefore may at least be partially due to their co-expression of Ii. It is therefore unlikely that constitutively class II^+ human tumor cells would be effective cell-based vaccines, unless their level of Ii were sufficiently reduced so that endogenous tumor antigen presentation could occur. Since induction of MHC class II expression by interferon-γ (23) or the class II transactivator gene (24) is accompanied by induction of Ii and HLA-DM (25-27), use of these agents to induce MHC class II expression by tumor cells is also unlikely to yield useful therapeutic cell-based vaccines.

The observation that class II-transfected tumor cells directly present antigen to $CD4^+$ T lymphocytes is in direct contrast to recent studies examining antigen presentation by tumors to $CD8^+$ T cells. In these studies, Huang and colleagues (28,29) examined class II^- tumors and demonstrated that in mice immunized with class I^- and/or B7-1^+ tumor cells, host-derived cells, and not tumor cells, are the exclusive APCs for tumor-encoded antigens. A clear dichotomy therefore exists between antigen presentation to $CD4^+$ and $CD8^+$ T cells, suggesting that different immunotherapeutic strategies need to be developed for activating these two T cell populations.

The studies presented in this and earlier reports (3,5, 30) indicate that cells expressing MHC class II/tumor peptide complexes and co-stimulatory molecules are potent inducers of tumor-specific immunity in mouse model systems. In a clinical setting, the use of autologous tumor cells as immunotherapeutic agents may be impractical, however. Our studies were originally under-

taken using intact tumor cells, because the relevant tumor antigens (peptides) are unknown. However, the isolation and characterization of tumor peptides are becoming increasingly common (31,32), and it may soon be feasible to design therapeutic strategies combining specific antigen presentation approaches, such as those described in this report, with known tumor peptides. Additional studies are clearly necessary to determine if carriers, such as liposomes, will induce immunity as effective as that induced by immunization with intact, genetically modified tumor cells.

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Cell-based vaccines for the stimulation of immunity to metastatic cancers

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Summary: We are developing vaccines for inducing immunity to metastatic cancers. Although primary tumors are frequently cured by surgery, chemotherapy, or radiation therapy, metastatic lesions often do not respond to these treatments or proliferate after conventional therapy is terminated. Vaccine therapy for established metastases as well as prophylactic vaccine treatment to prevent outgrowth of latent metastatic tumor cells would therefore be beneficial. Our goal is to activate $CD4^+$ and $CD8^+$ T lymphocytes; however, we have focused on activating tumor-specific $CD4^+$ T-helper lymphocytes because of their pivotal role as regulatory cells and in the generation of long-term immunological memory. The vaccines are based on the premise that tumor cells express potentially immunogenic antigens that could be targeted for T-cell activation, and that if appropriately genetically modified, tumor cells could be antigen presenting cells for these antigens. To facilitate direct antigen presentation to $CD4^+$ T cells, tumor cells have been transfected with syngeneic major histocompatibility complex class II, co-stimulatory molecule, and/or superantigen genes. *In vivo* studies in three mouse tumor models demonstrate that vaccination protects against future challenge with wild-type tumor, cures some solid primary tumors, reduces established metastatic disease, and extends mean survival time. Antigen presentation studies demonstrate that *in vivo* vaccine efficacy is directly related to *in vitro* antigen presentation activity. The relevance of antigen presentation activity of the vaccines is further confirmed by *in vivo* studies demonstrating that during the immunization process, the vaccines directly present tumor-encoded antigens to $CD4^+$ T lymphocytes. Adaptation of these vaccines for the treatment of human metastatic cancers is discussed.

Introduction

Activating the immune response against resident cancer cells has been a "dream" of immunologists since Ehrlich originally proposed his "magic bullet" strategy for targeting cytotoxic agents to tumor cells via tumor-specific antibodies. Although the concept of harnessing the immune system against autologous tumor is attractive, there has been over the years both skepticism and enthusiasm for cancer immunotherapy in the immunology community. During the past approximately 10 years, however, tumor immunology has undergone a renaissance, and there are now numerous experimental strategies that have demonstrated therapeutic efficacy in experimental animal models, and are in the process of being tested in clinical set-

tings. Much of the renewed interest in immunotherapy has resulted from the significant advances in understanding basic immunological phenomena and the ability, via the development of genetic engineering techniques, to manipulate the antitumor immune response.

The development of cancer vaccines has been a particularly active area of research. Proposed cancer vaccines could be used in several clinical settings: 1) tumor-free individuals at high risk of developing malignancies (e.g. individuals with genetic factors predisposing them to develop tumors) could be prophylactically immunized; 2) patients whose primary tumors only respond partially to conventional cancer therapies could be immunized following conventional therapy to inhibit growth of residual tumor cells; 3) patients whose primary tumors are completely cured, but who are at high risk of developing metastatic disease (e.g. breast cancer patients with malignant cells in the draining lymph nodes) could be immunized to protect against latent metastatic cancer; and 4) patients with tumors that are non-responsive to existing therapies (e.g. patients with dispersed metastatic tumor that does not respond to chemotherapy) could be given tumor vaccines as potential immunotherapy agents. Regardless of the setting in which cancer vaccines are administered, it is likely that they will be most effective in patients with minimal residual disease because many patients with advanced cancers and large tumor burdens are significantly immunosuppressed and therefore unlikely to mount an effective antitumor immune response (1).

Several characteristics of the immune response make it an ideal agent for selectively controlling tumor cell growth

Many primary tumors are localized and can be successfully treated with surgery and/or radiation therapy. Disseminated metastases and highly invasive tumors, however, are frequently not curable by surgery and/or radiation therapy, leaving chemotherapy as the principal method of treatment. A major problem with chemotherapy is its lack of tumor cell specificity and inability to localize to tumor cell deposits. As a result, chemotherapy, the major treatment for many metastatic cancers, cannot be used at high enough doses to completely destroy metastatic tumor because at these doses healthy tissue is also destroyed. (The same problem applies to the use of very high dose radiation therapy for the treatment of localized cancers when normal tissue intervenes between the tumor site and source of radiation.) In contrast, immunological effectors are active systemically because they are only toxic when they home and bind to their targets. In addition, the immune response exhibits acute immunological memory, making it capable of responding to its targets over a long period of time. Vaccination against malignant cells, therefore, has the potential to be a highly efficacious and specific treatment for metastatic cancer and may even provide long-term protection against recurrence of disease or latent outgrowth of pre-existing, quiescent lesions.

Effective vaccination against tumors requires the generation of both CD8+ and CD4+ T lymphocytes, with CD4+ T cells playing a key role in the immune response

The immune system mediates two basic types of responses: antibody-mediated (B-cell) responses and cell-mediated (T-cell) responses. Both responses are triggered by antigens, with T cells responding to fragments of antigen bound to integral membrane proteins encoded by the major histocompatibility complex (MHC), and B cells responding to intact, soluble antigen. Since many tumor antigens are cytosolic protein fragments bound to MHC molecules (2), T cells are particularly suited for targeting and destroying tumor cells. CD8+ effector T lymphocytes are potent cytotoxic cells, however, their activation and optimal activity frequently depend on co-activation of CD4+ T cells (3–7). Although the requirement for CD4+ T-cell help in non-tumor immune responses is well established, a role for CD4+ T-cell help in antitumor immunity has only recently been appreciated (8–13). Because of this pivotal role for CD4+ T lymphocytes in antitumor immunity, we have focused on generating cancer vaccines that specifically activate tumor-specific CD4+ T cells, and have hypothesized that improved CD4+ T-cell generation will lead to increased CD8+ T-cell activity resulting in enhanced tumor rejection and long-term protection against primary tumor recurrence and outgrowth of quiescent metastatic cells.

Although tumor antigens and tumor peptides have been identified, it is not clear which, if any, of these molecules are appropriate helper and target epitopes for antitumor immunity

A variety of tumor-associated, tumor-specific, and tumor-restricted antigens and peptides has been identified. Boon and colleagues pioneered the use of tumor-specific CD8+ T-cell clones to identify antigens on a variety of mouse and human tumors. These antigens fall into five categories: 1) normal unmutated self antigens expressed by tumor cells and expressed at lower levels by a restricted population of normal self cells (2, 14–16); 2) tissue-specific differentiation antigens that are overexpressed on tumor cells and expressed at lower levels on normal cells (17–22); 3) mutated self proteins that are uniquely expressed on tumor cells (17–26); 4) oncogenic proteins that are inappropriately expressed by tumor cells (27–29); and 5)

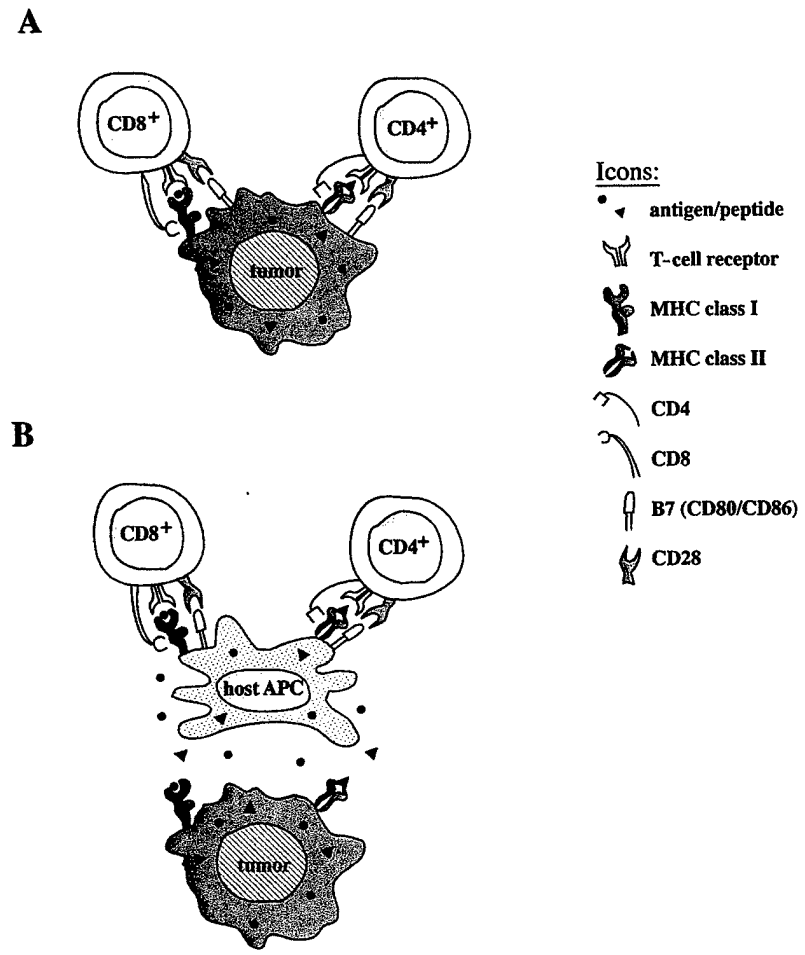

Fig. 1. Direct (A) and indirect (cross-priming) (B) pathways for activation of CD4+ and CD8+ T cells by MHC class II+CD80+ cell-based tumor vaccines.

virally encoded antigens (30). MHC class I tumor-associated peptides have also been identified using mass spectroscopy analysis of peptides that have been acid eluted from tumor cell encoded MHC class I molecules (30–33).

Many current cancer vaccine strategies use these peptides/antigens as immunogens and immunization is via inoculation of soluble peptide/antigen in adjuvant (34) or peptide/antigen pulsed on dendritic cells (35–39). Other immunization approaches use dendritic cells pulsed with total tumor cell RNA (40) or tumor cell extracts (41). Still other immunization approaches use tumor antigens cloned into viruses (42, 43). Although some of these vaccination approaches have led to tumor regression in experimental animal models and have stimulated antitumor immunity in patients, it is unclear if these defined antigens are necessary and sufficient to stimulate the optimal antitumor response. It is also unclear if generation of an immune response to a single or small number of epitopes is sufficient to stimulate a potent antitumor effect. Indeed, tumor-defined antigens will only be proven to be potent regression molecules when an immune response against them mediates total tumor rejection.

Rather than use the reductionist approach of immunizing with individual putative tumor antigens, we have focused on developing vaccine strategies which do not require specific identification of tumor antigens. We have reasoned that tumor cells *de facto* express the relevant regression antigens and therefore we are using intact tumor cells as the basic unit of the vaccines.

Results and discussion

We have designed a vaccine strategy based on the hypothesis that tumor cells can be genetically modified to directly present endogenous tumor antigens to CD4+ and CD8+ T cells and thereby stimulate potent tumor-specific immunity

Because most cells do not express MHC class II molecules, activation of tumor-specific CD4+ T cells normally occurs via indirect antigen presentation or cross-priming (*Fig. 1B*). During this

process professional antigen-presenting cells (APC) pinocytose soluble antigen (tumor antigen), process it in their endocytic compartment, and present it in the context of MHC class II molecules along with a co-stimulatory signal to CD4+ T cells. During the early stages of *in vivo* tumor growth, however, it is unlikely that soluble tumor antigen is available for uptake by professional APC because most tumor antigens are cytosolic or nuclear proteins and are not secreted by tumor cells (2, 15), and because tumor cells remain intact. Therefore, if soluble tumor antigen is not available, the cross-priming pathway for CD4+ T-cell activation is not accessible. We have hypothesized that by genetically modifying tumor cells to express MHC class II and costimulatory molecules, the tumor cells will directly present tumor antigen to CD4+ T cells. If the tumors also constitutively express MHC class I molecules, they will also directly present antigen to CD8+ T cells. Direct antigen presentation by tumor cells, as shown in *Fig. 1A*, by-passes the need for professional APC.

First-generation cell-based antitumor vaccines protected naive animals from a subsequent challenge of wild-type tumor cells

The first-generation cell-based antitumor vaccines were based on the hypothesis that tumor cells expressing syngeneic MHC class II molecules would directly present tumor-encoded antigens to CD4+ T cells and thereby facilitate antitumor immunity. We tested the hypothesis by transfecting the mouse A/J-derived SaI sarcoma (H-2^a) with syngeneic MHC class II genes (*Aa*^{k} and *Ab*^{k} genes; SaI/A^k tumor cells) and then used the transfectants to vaccinate tumor-free syngeneic A/J mice. The vaccinated mice were subsequently challenged with high doses of wild-type class II SaI tumor, and were 100% protected against tumor growth (9). *In vivo* antibody depletion studies confirmed that CD4+ T cells were required for the protection. The ability of class II-transfected autologous tumor to vaccinate naive mice against subsequent wild-type tumor challenge was confirmed by other groups using similar approaches (44, 45).

Other investigators (46) demonstrated that antigen presentation by MHC class II molecules required the cytoplasmic domain of the class II heterodimer. Because our hypothesis was that the class II-transfected tumor cells function as APC for tumor antigens, we speculated that tumor cells transfected with MHC class II genes truncated for their cytoplasmic domains would not be immunogenic and would not stimulate antitumor immunity. We tested this hypothesis by transfecting SaI sarcoma cells with genes encoding *Aa*^{k} and *Ab*^{k} chains truncated for 12 and 10 amino acids, respectively at their carboxyl (cytoplasmic) ends (SaI/A^k/tr cells). As expected, these transfectants were not effective vaccines and were tumorigenic themselves (47). However, the vaccine effect was restored if the SaI/A^k/tr cells were further transfected with the gene encoding the CD80 (B7.1) co-stimulatory molecule (SaI/A^k/tr/B7.1 transfectants) (48). This result was consistent with the concept that T-cell activation required two signals, an antigen-specific signal delivered via the MHC/peptide complex of the APC to the T-cell receptor (TCR) of the T cell, and a second signal delivered by a co-stimulatory molecule such as CD80 and/or CD86 on the APC to the C28 receptor on the T cell (49).

These experiments, along with two other studies (50, 51) demonstrated that tumor cell expression of co-stimulatory molecules enhanced antitumor immunity and also suggested that one of the functions of MHC class II molecules in antigen presentation is induction of CD80 in the APC. Subsequent experiments assessing CD80 and CD86 expression by SaI/A^k vaccines *in vivo* during the immunization period corroborated the hypothesis that CD80 and CD86 expression are essential for priming an antitumor immune response and that the MHC class II-transfected tumor cells are induced to express these molecules during the immunization period (52). Subsequent experiments in other vaccine/tumor systems corroborated the requirement for co-stimulatory molecule expression, however these studies found that CD80 and/or CD86 expression were induced in both vaccinating tumor cells and host-derived APC (53).

In collaborative studies with the lab of L. Glimcher, site-directed mutagenesis was used to generate mutations in the cytoplasmic region of the *Ab*^{k} gene to identify which amino acids are critical for antigen presentation, tumor rejection, and induction of co-stimulatory molecules. These studies demonstrated that amino acids G and P at positions 226 and 227, respectively, are essential for antigen presentation, tumor rejection, and induction of CD80 and CD86 in the vaccines (154) (V. Clements, S. Ostrand-Rosenberg, unpublished result). This region of the cytoplasmic tail of the MHC class II β chain may contain a signaling motif or associated signaling molecules that lead to regulation of CD80 and CD86 expression and/or other molecules critical for antigen presentation (46). This hypothesis appears unlikely, however, because the class II cytoplasmic tail is short and attempts to identify signaling motifs or associated enzymes have been unsuccessful.

Second-generation vaccines, consisting of tumor cells transfected with syngeneic MHC class II and CD80 genes, are effective vaccines for the treatment of mice with established primary sarcoma and metastatic mammary carcinoma

The first-generation vaccines were tested in tumor-free mice for their ability to protect against subsequent challenge with

Table 1. Summary table of mice cured of established wild-type SaI/N tumors after therapy with SaI/A^k/B7.1 vaccine

Dose of transplanted SaI/N tumor[a]	Age of SaI/N primary tumor at initiation of therapy (days)	Therapeutic vaccine	No. of mice cured/total treated
10^5–3.3 × 10^5	–	–	0/11
	9–13	SaI/A^k/B7.1	9/15
	9	SaI/B7.1	0/10
10^6	–	–	0/14
	5–19	SaI/A^k/B7.1	19/33
	9	SaI/B7.1	0/10

[a] Mice were inoculated with live SaI/N cells subcutaneously in the flank with the indicated number of cells and the tumors allowed to grow progressively. A single treatment with the vaccine was given on the indicated day. Tumors ranged between 2 mm and 7 mm at the start of therapy. Mice were tallied as "cured" if their tumors completely regressed and the mice remained tumor-free for the 3-month duration of the experiment.

wild-type tumor. Vaccines could be used in this setting for the prophylactic vaccination of tumor-free individuals who are at high risk of developing tumors, however, they could also be used as therapeutic agents for treating established tumors that are non-responsive to existing therapies. Many metastatic cancers are in this latter category. To model this clinical situation more closely, the second-generation vaccines, consisting of tumor cells transfected with MHC class II plus CD80 or CD86 genes, were tested in mice carrying established primary, solid tumors and mice with established metastatic disease.

In the sarcoma model, A/J mice were inoculated substaneously (s.c.) in the flank with 10^5–10^6 wild-type SaI tumor cells and the tumors allowed to grow until they were 2–7 mm in diameter and well vascularized (approximately 1.5–3 weeks after initial inoculation). Therapy was then initiated (one injection/week of 10^6 SaI/A^k/B7.1 transfectants × 3 weeks) and tumor growth monitored. As shown in *Table 1* which is a summary of these experiments, tumors in approximately 55% of treated mice completely and stably regressed when treated with SaI/A^k/B7.1 vaccines. In contrast, treatment with single transfectants (SaI/A^k or SaI/B7.1 vaccine) had no affect on tumor growth. The double transfectant (SaI/A^k/B7.1 vaccine), therefore, is significantly more effective than vaccines expressing only MHC class II or B7.1 (55). Studies by Chen and co-workers (56) demonstrated that vaccines consisting of tumor cells transfected with B7 were only effective if the tumor cells were inherently immunogenic. Because SaI is a relatively poorly immunogenic tumor, the results suggest that even poorly immunogenic tumors can be converted to effective vaccines provided the vaccine co-expresses B7 and syngeneic MHC class II.

The efficacy of the MHC class II vaccines against metastatic disease was tested using the BALB/c-derived (H-2^d) 4T1 mammary carcinoma (57). This mouse tumor system is a very appropriate model for human mammary cancer, because 4T1 tumor cells migrate from the primary tumor site (the mammary fat pad) to virtually all organs to which human mammary tumors spread. Within 2 weeks of inoculation of as few as 7 × 10^3 cells into the mammary fat pad, metastatic cells can be found in the draining lymph nodes and lungs, and within 3–4 weeks, tumors cells are present in the spleen, liver, and brain (58). Inoculation of irradiated unmodified 4T1 tumor cells does not stimulate any antitumor response, demonstrating that 4T1 is very poorly immunogenic. To test the efficacy of 4T1-based vaccines, tumor cells were transfected with syngeneic MHC class II Aa^d, Ab^d and/or CD80 genes (4T1/A^d, 4T1/B7.1 transfectants) and the resulting transfectants used to treat female BALB/c mice with established primary tumors. Therapy consisted of injections of irradiated transfectants twice a week for 4 weeks and was started 9–14 days after the mice had received 7 × 10^3 wild-type tumor cells in the mammary fat pad. The primary tumors ranged in size from approximately 2–5 mm in diameter at the start of therapy. Since 4T1 cells are resistant to 6-thioguanine (57), the number of metastatic cells in an organ can be quantified by plating out dissociated explanted organs in medium supplemented with 6-thioguanine and counting the number of tumor cell colonies after 10 days of culture. After 4 weeks of vaccine therapy (6 weeks after initial tumor cell inoculation), mice treated with 4TI/A^d plus 4TI/B7 transfectants had significantly fewer metastatic cells in the lung than untreated mice or mice treated with either transfectant alone. Tumor challenge experiments in BALB/c nu/nu mice confirmed that T cells were involved in the antitumor effect (58).

Similar experiments have also been performed using the C57BL/6-derived B16melF10 melanoma tumor in which mice

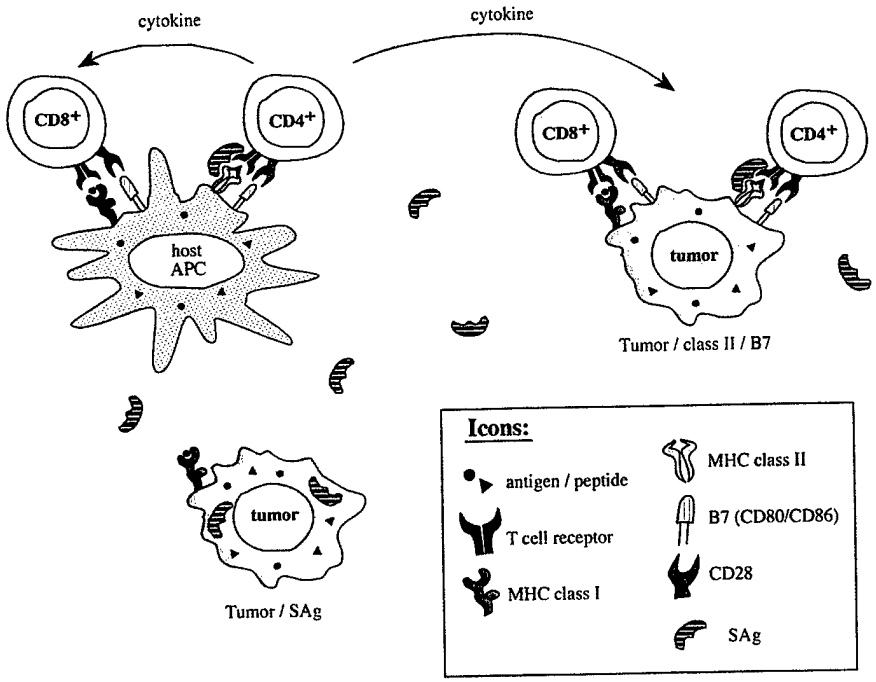

Fig. 2. Schematic diagram showing the proposed mechanism of CD4+ T-cell activation by the MHC class II, CD80 plus SEB vaccines. Tumor-specific CD4+ T cells will be directly activated by the cell-based vaccines expressing MHC class II plus CD80. These CD4+ T cells will then receive additional activation signals when SEB binds to the side of the MHC class II/peptide/TCR complex bridging the APC and the responding T cell. Secreted SEB will also enhance the activation of T cells activated by professional APC.

carrying i.v.-induced lung metastases were treated with melF10 transfectants (59, 60). In the B16melF10 tumor system as in the SaI and 4T1 tumor systems, the most dramatic antitumor effect was seen following vaccination with the class II+B7+ transfectants.

Third-generation vaccines, consisting of tumor cells transfected with syngeneic MHC class II, CD80, and superantigen *Staphylococcus aureus* enterotoxin B (SEB) genes, are the most effective agents for the treatment of mice with established metastatic disease

Although therapy with the second-generation vaccines dramatically reduces metastatic disease in the mammary carcinoma system, many of the treated mice still contained metastases. We therefore reasoned that the existing vaccines activated CD4+ and CD8+ T cells, however, better activation might lead to enhanced antitumor immunity. We therefore incorporated a gene encoding a superantigen (sAg) into the vaccines. SEB is a potent activator of CD4+ T cells (61) that bridges the TCR of the responding T cells and the MHC class II molecule of the APC by binding along the side of the TCR/MHC II complex. Although CD4+ T-cell activation by SEB is not antigen-specific, we reasoned that addition of SEB to the MHC class II/B7.1 vaccine would provide additional activation signals to these CD4+ T cells that have been activated in an antigen-specific fashion by the MHC class II+B7.1+ vaccine. To test this hypothesis, we generated SEB-transfected 4T1 cells (4T1/SEB) and combined these cells with 4T1/A^d/B7 cells as a third-generation vaccine. Fig. 2 is a diagram of the hypothesized action of the third-generation vaccine.

The vaccines were tested in BALB/c mice with established primary and metastatic 4T1 mammary carcinomas. Mice were inoculated in the mammary fat pad with 7×10^3 wild-type 4T1 tumor cells and when the primary tumors reached approximately 1–4 mm in diameter (2 weeks after initial inoculation), twice weekly therapy injections of 10^6 irradiated transfectants were given for 4 weeks. The mice were then sacrificed and the number of metastatic tumor cells in the lungs measured using the clonogenic assay. Only mice treated with the combination vaccine of 4T1/A^d/B7 plus 4T1/SEB cells had significantly fewer lung metastases than mice treated with the wild-type control cells, suggesting that the class II+B7.1+ vaccine synergized or functioned additively with the SEB vaccine (B. A. Pulaski, S. Ostrand-Rosenberg, unpublished result).

Following surgical removal of primary tumor, treatment with the class II/B7/SEB vaccine significantly extends mean survival time

In the previous experiments the MHC class II/B7.1/SEB vaccine was used to treat mice with primary tumor plus metastatic disease. Many primary tumors, however, including melanoma and mammary carcinoma are curable by surgical resection, so

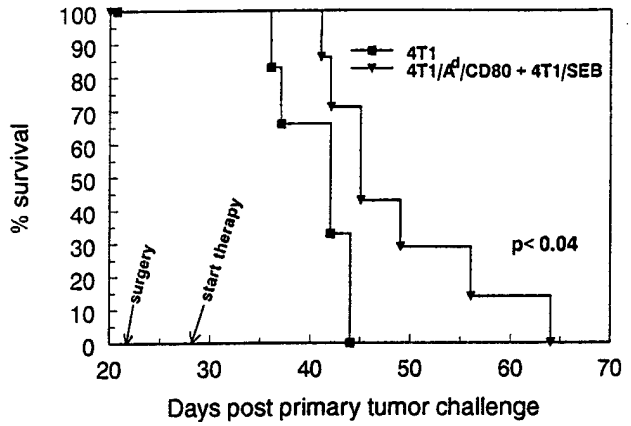

Fig. 3. Mice with established metastatic disease have extended mean survival time following treatment with the 4T1/A^d/B7 + 4T1/SEB vaccine. Mice were inoculated with wild-type 4T1 tumor in the mammary fat pad and the tumors surgically resected 3 weeks later when they were 3–6 mm in diameter. Therapy of 4T1/A^d/B7 + 4T1/SEB or 4T1 alone was begun one week after surgery, and the mice followed for survival. The survival times between the two treatment groups are significantly different ($p < 0.04$ using the Student's t-test).

it is unlikely that vaccine therapy would be performed in patients with existing primary tumor. Anticancer vaccines are more likely to be administered after elimination of primary tumor when the risk of recurrence of primary tumor or outgrowth of metastatic tumor cells is high. To model this clinical situation, we have tested the vaccines in a post-surgery setting using the following experimental design: BALB/c mice were inoculated in the mammary fat pad with 7×10^3 wild-type 4T1 tumor cells and the tumors were allowed to grow progressively for approximately 3 weeks, at which time the average tumor diameter (TD) was 5.2 ± 0.3 mm. Primary tumors were then surgically removed. Therapy of one injection per week of irradiated 4T1/A^d/B7 plus 4T1/SEB or 4T1 cells alone was started one week after surgery and continued throughout the experiment. As shown in *Fig.* 3, mice treated with the 4T1/A^d/B7 plus 4T1/SEB vaccine have a significantly extended mean survival time as compared to mice treated with 4T1.

Previous studies established that at week 4, when therapy was started 91%, 82% and 36% of mice had metastatic cells in their lungs, liver, and brain, respectively, and that the range of metastatic cells for these organs was 6–250,000, 7–7,800, and 1–116, respectively (58). Administration of the therapeutic vaccines, therefore, significantly extends mean survival time of animals that have very advanced, established metastatic disease at the initiation of therapy.

MHC class II-transfected, B7.1-transfected tumor cell vaccines induce antitumor immunity because they directly present endogenously synthesized tumor antigen(s) to CD4$^+$ T cells

Our hypothesis for the efficacy of the tumor vaccines is that the transfected tumor cells directly present endogenously synthesized tumor antigens to CD4$^+$ T cells and thereby enhance tumor-specific CD4$^+$ T-cell activation (9,11). This hypothesized mechanism is somewhat unconventional for several reasons: 1) presentation of MHC class II-restricted antigens is usually via professional APC such as dendritic cells, macrophages, or B lymphocytes which have additional "accessory" molecules that facilitate antigen presentation (62, 63); 2) MHC class II molecules of professional APC usually present exogenously synthesized antigens (63); and 3) previous studies assessing antigen presentation of MHC class I-restricted antigens to CD8$^+$ T cells established that even endogenously synthesized antigen is not usually presented by the cells synthesizing the antigen, but rather by professional APC that pinocytose and present the antigen by "cross-priming" or "indirect" antigen presentation (64, 65).

To determine if the cell-based vaccines induce antitumor immunity because they function directly as APC for endogenously encoded tumor antigens, we have used the experimental design shown in *Fig.* 4. (C57BL/6 X A/J)F1 mice were lethally irradiated and reconstituted with either C57BL/6 or A/J bone marrow [C57BL/6 → (A/J X C57BL/6)F1 or A/J → (A/J X C57BL/6)F1]. The resulting chimeras have exclusively C57BL/6 (I-A^b) or A/J (I-A^k) APC, respectively. These chimeras are challenged with either SaI/A^k/hen egg lysozyme (HEL) or SaI/A^b/HEL tumor cells and the MHC restriction pattern of the HEL-specific CD4$^+$ T cells assessed. If the tumor cells are the predominant APC, then the HEL reactivity will be restricted to the genotype of the tumor. If antigen presentation is via cross-priming, then the CD4$^+$ response will be restricted to the genotype of the bone marrow donor. *Fig.* 5 shows a representative experiment performed at 1 week after vaccination. Regardless of the genotype of the tumor (either SaI/A^k/HEL or SaI/A^b/HEL tumor cells), the tumor-encoded class II genotype is the dominant genotype for HEL presentation, and the host genotype is the minor genotype. Similar results were obtained using SaI/A^k/B7/HEL and SaI/A^b/B7/HEL-transfected tumor cells into C57BL/6 → (A/J X C57BL/6)F1 chimeras and A/J → (A/J X C57BL/6)F1 chimeras, respectively (V. Clements, S. Ostrand-Rosenberg, unpublished results). These results indicate that during the initial stage of vaccination, the genetically modified tumor cell is the principal APC and that cross-priming or indirect antigen presentation occurs, but is a relatively minor contributor to the antitumor response (66, 67).

In contrast to presentation of MHC class II-restricted tumor antigens, presentation of MHC class I-restricted tumor antigens via cytokine modified tumor cells is principally indirect and via cross-priming. For example, granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-3-modified tumor cells stimulate an antitumor immune response via host-derived APC rather than by the genetically modified tumor cells themselves (68–70). CD80 (B7.1)-modified tumor cell vaccines stimulate $CD8^+$ cells either by functioning directly as APC for tumor-encoded antigens (70), or via cross-priming (71).

The apparent discrepancy between antigen presentation pathways of the class II vaccines vs the class I vaccines is puzzling. We initially proposed that the differences between the class I and class II vaccines could be due to the kinetics of the antitumor response (66). Early on after vaccination there may be very little soluble tumor antigen (class I or class II-restricted epitopes) available for uptake by professional APC. However, as the antitumor immune response continues, tumor cells will be destroyed and soluble antigens may become available for uptake by professional APC. Because the class II studies were performed early during immunization (1 week after immunization), while the class I studies were performed 2 or more weeks after immunization, the differences may reflect variations in the response kinetics. However, we have assessed antigen presentation by the SaI/A^k/B7/HEL vaccines in recent experiments and found that even 3 to 5 weeks after vaccination, the predominant HEL response is still restricted to the genotype of the tumor cell, indicating that the tumor cell vaccine itself remains the principal APC (V. Clements, S. Ostrand-Rosenberg, unpublished results).

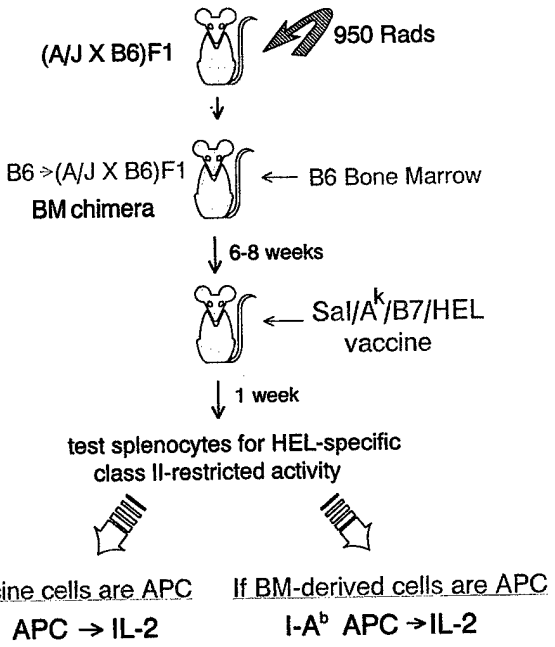

Fig. 4. Experimental design for the bone marrow chimera experiments identifying the APC for tumor-encoded endogenous antigen. (A/J X C57BL/6)F1 mice ($H\text{-}2^a/H\text{-}2^b$; $I\text{-}A^k/I\text{-}A^b$) were lethally irradiated and reconstituted with A/J or C57BL/6 bone marrow (BM) [A/J → (A/J X C57BL/6)F1 or C57BL/6 → (A/J X C57BL/6)F1 chimeras, respectively.] Six to eight weeks after bone marrow reconstitution, the chimeras were challenged with SaI/A^k/HEL, SaI/A^k/B7/HEL, SaI/A^b/HEL, or SaI/A^b/B7/HEL tumor cells. Seven days after tumor challenge, spleens were removed and tested for IL-2 release in response to A/J or C57BL/6 APC plus HEL.

MHC class II-transfected tumor cells efficiently present endogenously synthesized tumor antigens to $CD4^+$ T lymphocytes provided they do not co-express the class II-associated accessory molecule, invariant chain

Fig. 6 shows a simplified diagram of the intracellular trafficking pathways of MHC class I and class II molecules during antigen processing and presentation. As shown in this diagram, proteins synthesized within the APC are degraded in the proteosome and transported via the TAP transporters into the endoplasmic reticulum (ER) where they can be bound by newly synthesized MHC class I molecules. The class I/peptide complexes then traffic to the Golgi and via the default secretory pathway to the cell surface where they are anchored into the plasma membrane via their hydrophobic region (72, 73). In contrast, MHC class II molecules do not bind peptides in the ER. Instead, MHC class II molecules bind a protein called the invariant chain (Ii) in the ER. Ii and class II genes are co-ordinately transcriptionally regulated so that newly synthesized class II molecules are always co-expressed with newly synthesized Ii molecules. Because Ii chain binds to the peptide binding cleft of the class II molecule, peptides within the ER cannot bind to class II molecules in the ER. The Ii chain contains a trafficking signal that directs the class II/Ii complex to an endocytic compartment. In the early endosome the pH drops and the Ii chain begins to degrade. As the endosomal compartment becomes more acidic, more of the Ii chain degrades leaving only a small portion, termed the class II-associated Ii peptide (CLIP), bound to the antigen-binding cleft of the class II heterodimer. Concurrently, the APC has pinocytosed exogenously synthesized antigen which is degraded into peptides within parallel endocytic vesicles. Eventually the endocytic vesicles containing exogenous antigen merge with the vesicles containing class II/ CLIP complexes (74, 75). Within these so-called MIIC compartments, CLIP is displaced by processed exogenous peptides with the help of the class II-like molecule H-2M (HLA-DM) (62, 63,

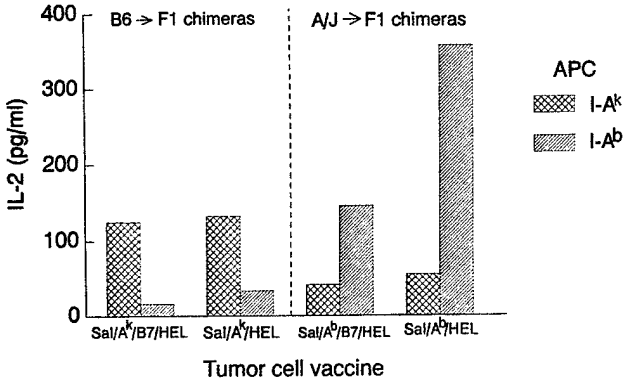

Fig. 5. Tumor cells are the predominant APC for tumor antigen presentation to CD4+ T cells. C57BL/6 → (A/J X C57BL/6)F1 or A/J → (A/J X C57BL/6)F1 bone marrow chimeras were immunized i.p. with Sal/A^k/HEL or Sal/A^b/HEL cells, respectively or Sal/A^k/B7/HEL or Sal/A^b/B7/HEL cell, respectively, and splenic T cells assayed 1 week later for IL-2 production in response to HEL presented by I-A^k vs. I-A^b.

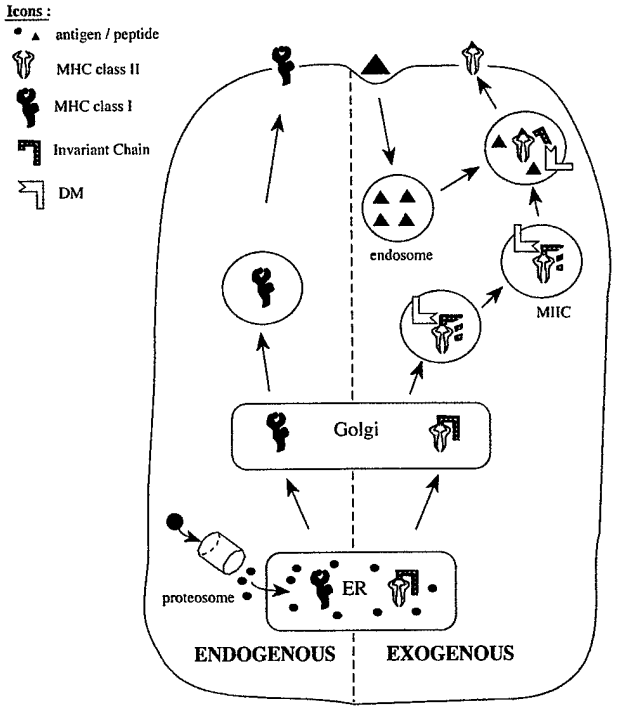

Fig. 6. Antigen-processing and presentation pathways for MHC class I and MHC class II-restricted antigens. MHC class I molecules typically present endogenously synthesized antigens, while MHC class II molecules typically present exogenously synthesized antigens due to co-expression of invariant chain and DM.

76). The end result of these trafficking pathways is that MHC class I molecules usually present endogenously synthesized antigens, while class II molecules usually present exogenously synthesized antigens.

The class II-modified tumor cell vaccines were designed so that endogenously synthesized tumor antigens are presented by the transfected MHC class II molecules. We reasoned that in the absence of Ii chain, proteosome-degraded cellular proteins which are potential tumor antigens could be bound by newly synthesized class II molecules in the ER. To test this hypothesis, we generated tumor cells expressing different combinations of syngeneic MHC class II, Ii and/or DM proteins. SaI sarcoma cells expressing class II plus Ii were generated by transfecting Sal/A^k cells with the genomic Ii gene (Sal/A^k/Ii cells). Sarcoma cells expressing class II, Ii, and DM were generated by transducing SaI cells with the class II transactivator (CIITA), a regulatory gene that coordinately upregulates class II, Ii and DM (SaI/CIITA cells) (77–82). To determine the effect of these molecules on presentation of endogenous antigen, the vaccines were further transfected with the *HEL* gene and their ability to present HEL to an I-A^k-restricted HEL-specific T-cell hybridoma measured. As shown in the summary *Table* 2 only Sal/A^k cells present endogenously synthesized HEL, confirming that Ii expression blocks endogenous antigen presentation. As expected, only cells containing Ii present antigen when the vaccines are fed exogenous HEL (59, 83).

The genomic Ii gene transcribes several forms of Ii protein. In the mouse there are two major functional forms: p31 and p41. It has been reported that the two forms have different functions and that p41 is the predominant form that affects presentation of exogenous antigen (84). In the Sal/A^k/Ii transfectants used in *Table* 2, the p31 and p41 forms were approximately equally expressed (85). To determine if p31 and p41 have differential effects on presentation of exogenous antigen by class II, Sal/A^k cells were further transfected with cDNA constructs encoding either p31 or p41 (Sal/A^k/p31, Sal/A^k/p41

cells). Antigen presentation assays using 4–6 clones of each transfectant and exogenous HEL demonstrated that both p31 and p41 expression facilitated exogenous antigen presentation, although p41 was slightly more effective. This result confirms previous studies for the effects of p41. These results, however, do not agree with the earlier studies which showed that p31 did not enhance exogenous antigen presentation (L. Hanyok, S. Ostrand-Rosenberg, unpublished results).

Expression of H-2M (DM) enhances presentation of exogenous antigen by the vaccines, but does not alter presentation of endogenous antigen

When MHC class II molecules present exogenous antigen, DM expression facilitates the loading of antigen (86–88). We have generated DM transfectants to determine if DM plays a similar role in presentation of endogenous antigen. SaI sarcoma cells were transfected with the H-2M (DM) gene and in some cases with the HEL gene as an endogenous antigen, and the ability of the transfectants to present antigen to an I-A^k-restricted, HEL-specific T-cell hybridoma assessed. As shown in *Table* 2 expression of DM had no effect on endogenous HEL presentation, while it had the predicted profound effect on presentation of exogenously fed HEL.

In vitro antigen presentation capacity is predictive of vaccine efficacy

Because the vaccines function as APC for endogenously encoded tumor antigens, we hypothesized that the *in vitro* APC activity of the transfectants would be predictive of their vaccine efficacy. To test this hypothesis, the various transfectants were inoculated into semi-syngeneic mice and their tumorigenicity was evaluated. As shown in *Table* 3, SaI sarcoma cells that express class II without co-expression of Ii are immunogenic (SaI/A^k), while tumor cells co-expressing class II plus Ii, or class II plus Ii plus DM (SaI/A^k/Ii or SaI/CIITA) remain tumorigenic (83). Co-expression of DM does not alter tumorigenicity (L. Qi, S. Ostrand-Rosenberg, unpublished results). These results indicate that the *in vitro* antigen presentation activity of the vaccines is highly predictive of their vaccine efficacy *in vivo*, and confirm that the optimal vaccines for activation of $CD4^+$ T cells will express MHC class II molecules and not co-express Ii chain.

These results also explain why tumors that constitutively express MHC class II molecules (e.g. B-cell lymphomas, some melanomas) are not inherently more immunogenic. Because class II and Ii are co-ordinately regulated, tumors that constitutively express class II co-express Ii (59). These tumors, therefore, do not present endogenously synthesized tumor antigens and would not be expected to be more immunogenic than class II^- tumors. Likewise, therapy with interferon (IFN)-γ or transduction with the CIITA would not be expected to increase tumor cell immunogenicity because these agents upregulate both class II and Ii expression (83).

Adapting this vaccination approach for the treatment of human tumors

For the mouse studies we have used autologous tumor cells as the "base" vaccine. The tumor cells are established cell lines and are stable and readily transfectable with syngeneic MHC class II genes, co-stimulatory molecule genes, and superantigen genes. Although autologous human tumor cells have been used in clinical trials (http: //cancernet.nci.nih.gov), the large scale clinical use of autologous human tumor material presents several significant problems. 1) Primary human tumor material can be technically difficult to obtain and maintain in culture and hence its adaptation as a vaccine may be technically problematic. 2) Human tumor cells are frequently not phenotypically or genetically stable in culture and therefore may not be suitable for the necessary *in vitro* manipulation. 3) Not all human tumors are readily transfectable, transducible, or infectable. 4) Generation of the tumor cell vaccines takes a finite amount of time, and patients with cancer may require therapy before the vaccines are ready. 5) Obtaining primary human material may be very costly and will be specific to individual patients. Such a customized therapy may not be cost-effective for large numbers of patients. Because of these potential limitations, we are exploring sources other than autologous tumor for human cell-based vaccines.

One possible alternative approach is to use established human tumor cell lines as the "base" lines for the vaccines. For example, vaccines for the treatment of breast cancer, could consist of a panel of established human breast cancer lines. Because tumor antigens appear to be shared among subsets of tumors (2, 15) it is likely that a panel of 6 or more breast tumors will contain at least 1 or more tumor antigens expressed by a patient's breast tumor. (e.g. the her2/neu tumor antigen is over-expressed by approximately 20–30% of human breast tumors (89)). To activate tumor antigen-specific $CD4^+$ T cells, each cell line of the panel would be transfected with a common *HLA-DR* gene. Patients to be treated with the vaccine would be HLA-DR typed and then immunized with the vaccine panel expressing the same HLA-DR as the patient. This process should stimulate HLA-DR-restricted $CD4^+$ T-cell responses to several of the patient's tumor antigens.

Results from the mouse studies demonstrate that candidate "base" cell lines for the vaccines must meet the following criteria: 1) they must not constitutively express invariant chain

Table 2. Only MHC class II$^+$Ii$^-$ tumor vaccines present endogenously synthesized antigen

Tumor cell vaccine	Presentation of antigen	
	Endogenous antigen	Exogenous antigen
Sal	–	–
Sal/A^k	+++	–
Sal/A^k/Ii	–	–
Sal/CIITA		
(Sal/A^k/Ii/DM)	–	+++
Sal/A^k/DM	+++	++++

Genetically modified tumor cells (vaccines) were either pulsed with exogenous hen egg lysozyme (HEL; exogenous antigen) or transfected with the *HEL* gene (endogenous antigen). APC (vaccines) were incubated with the I-A^k-restricted HEL46-61-specific 3A9 T-cell hybridoma and IL-2 release measured by enzyme-linked immunosorbent assay (ELISA).

Table 3. Expression of invariant chain, but not DM, blocks vaccine efficacy of MHC class II$^+$ tumor vaccines

Vaccine[a]	Tumorigenicity
Sal	+++
Sal/A^k	–
Sal/A^k/Ii	+++
Sal/CIITA	
(Sal/A^k/Ii/DM)	+++
Sal/A^k/DM	–

Mice were challenged i.p. with the indicated cell-based tumor vaccines and followed for tumor incidence.

because Ii chain expression inhibits presentation of endogenous tumor antigen; 2) they must not be induced by IFN-γ to express Ii because IFN-γ levels *in vivo* during immunization might induce Ii and thereby inhibit the antigen presentation phenotype of the vaccine; 3) they must readily proliferate in culture and stably maintain their phenotype so they can be expanded for immunization; and 4) they must be easily transfectable so we can transfect and express MHC class II, CD80, and *sAg* genes.

Using established cell lines as the "base" vaccine would not only provide many of the benefits of autologous vaccines, but may provide additional advantages: 1) It is not necessary to biochemically characterize individual class II-restricted tumor antigens because the immunizing panel will express many tumor antigens and it is statistically likely that there will be overlap with antigens expressed by the patients' tumors. 2) Although the vaccine panel will be HLA-DR matched to the patients, the vaccines will also express many allogeneic MHC class I molecules. Studies have demonstrated that MHC class I alloantigen expression can provide an "adjuvant" effect and stimulate tumor-specific immunity (90). The vaccine panel, therefore, may induce adjuvant effects in addition to the class II-restricted effect. 3) Because the vaccines can be developed in advance, patients need only be HLA-DR typed to determine which set of vaccines to use. 5) The ready availability of the panel will minimize the amount of time for getting patients onto a vaccine protocol.

Although successful therapies in experimental animals frequently do not translate into successful therapies in patients, the mouse studies suggest that the cell-based vaccines described here may have some utility for the treatment and/or prevention of metastatic cancer. Clearly there are challenges and potential problems in adapting this cell-based vaccine therapy for the treatment of human tumors. Only through clinical trials and additional studies measuring antitumor immune responses following vaccination can we ultimately determine if this cell-based vaccination strategy will have any efficacy for the treatment of human metastatic cancers.

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MHC Class II Presentation of Endogenous Tumor Antigen by Cellular Vaccines Depends on the Endocytic Pathway but not H2-M

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We have developed cell-based cancer vaccines that activate anti-tumor immunity by directly presenting endogenously synthesized tumor antigens to CD4+ T helper lymphocytes via MHC class II molecules. The vaccines are non-conventional antigen-presenting cells because they express MHC class II, do not express invariant chain or H-2M, and preferentially present endogenous antigen. To further improve therapeutic efficacy we have studied the intracellular trafficking pathway of MHC class II molecules in the vaccines using endoplasmic reticulum-localized lysozyme as a model antigen. Experiments using endocytic and cytosolic pathway inhibitors (chloroquine, primaquine, and brefeldin A) and protease inhibitors (lactacystin, LLnL, E64, and leupeptin) indicate antigen presentation depends on the endocytic pathway, although antigen degradation is not mediated by endosomal or proteasomal proteases. Because H2-M facilitates presentation of exogenous antigen via the endocytic pathway, we investigated whether transfection of vaccine cells with H-2M could potentiate endogenous antigen presentation. In contrast to its role in conventional antigen presentation, H-2M had no effect on endogenous antigen presentation by vaccine cells or on vaccine efficacy. These results suggest that antigen/MHC class II complexes in the vaccines may follow a novel route for processing and presentation and may produce a repertoire of class II-restricted peptides different from those presented by professional APC. The therapeutic efficacy of the vaccines, therefore, may reside in their ability to present novel tumor peptides, consequently activating tumor-specific CD4+ T cells that would not otherwise be activated.

Key words: endogenous antigen presentation, H-2M, immunotherapy, intracellular trafficking, MHC class II, tumor vaccines

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Tumor cells display a wide variety of antigens recognized by T lymphocytes and which could serve as tumor rejection molecules (1,2). Tumor-bearing individuals, however, frequently do not generate an effective immune response against these molecules and, as a result, the host's immune system may not limit autologous tumor growth. To facilitate the generation of anti-tumor immunity, cancer vaccines are being developed to stimulate T cell responses to tumor antigens. Because of the central role for CD4+ helper T cells in the anti-tumor response (3–8), we have focused on developing vaccines that augment tumor-specific CD4+ T cells. The vaccines have been tested in three independent mouse tumor models (sarcoma, melanoma, and mammary carcinoma) and they have significant therapeutic efficacy against pre-established primary tumor and metastatic disease (9–11, B. Pulaski and S. Ostrand-Rosenberg, submitted).

The vaccines use autologous tumor cells transfected with syngeneic MHC class II and costimulatory molecule genes and are based on the hypothesis that the genetically modified tumor cells are antigen-presenting cells (APC) for endogenously synthesized tumor antigens (5,6,12,13). Studies using chimeric mice confirmed that the genetically modified tumor cells are the predominant APC following vaccination, and that cross-priming is only minimally involved (14,15), while antigen presentation studies confirmed that the transfected class II molecules present endogenously synthesized molecules and that vaccine efficacy closely correlates with vaccine antigen presentation capacity (16). The vaccine cells, therefore, are somewhat unusual in that they efficiently present endogenously synthesized antigen, while professional APC preferentially, although not exclusively, present exogenously synthesized class II peptides (17,18).

Although previous studies have examined endogenous MHC class II antigen presentation by both professional and non-professional APC (19–21), the vaccine cells are atypical APC because they do not express invariant (Ii) chain or H-2M, accessory molecules that facilitate antigen presentation via the endocytic route (22–24). In the absence of Ii and/or H-2M, it is unclear where class II molecules bind endogenously synthesized peptides or if class II/peptide complexes assemble via the endocytic or cytosolic pathway. To identify the intracellular pathway taken by class II molecules in the vaccines, we have transfected the vaccine cells with the model antigen, hen eggwhite lysozyme (HEL) localized to the endoplasmic reticulum (erHEL), and assessed presentation of HEL in the presence of drugs that selectively block the cytosolic and/or endocytic pathways and/or inhibit enzymes restricted to these pathways. The drug experiments indicate that in the vaccines, class II molecules traffic via the classical endocytic pathway. Because H-2M is a critical protein for presentation of exogenously synthesized antigen processed via the endocytic route (24), we have speculated it may also affect presentation of class II-restricted endogenously synthesized antigen in the absence of Ii chain. In contrast to exogenous antigen presentation, however, H-2M does not

affect endogenous antigen presentation by MHC class II, and its co-expression in MHC class II-transfected tumor vaccines does not alter vaccine efficacy.

Results

Chloroquine, primaquine, and brefeldin A inhibit presentation

To clarify the intracellular trafficking pattern of MHC class II molecules and endogenous antigen in the MHC class II-transfected tumor cell vaccines, antibiotics that selectively block components of the antigen presentation pathways have been used. As shown in Figure 1, chloroquine, which inhibits endosomal acidification, has no effect on the presentation of class I-restricted endogenous antigen via the cytosolic pathway (EL-4 cells transfected with ova, presenting antigen to the K^b-restricted, chicken ovalbumin (OVA) (256–264)-specific B3Z $CD8^+$ T cell hybridoma), but is a potent inhibitor of the endocytic pathway (25) for presentation of class II-restricted exogenous antigen (TA3 cells pulsed with exogenous HEL, presenting antigen to the I-A^k-restricted HEL (46–61)-specific 3A9 $CD4^+$ T cell hybridoma), and for presentation of endogenous HEL by the tumor vaccine (Sal/A^k/HEL cells with 3A9 hybridoma). Similar results were obtained with primaquine, another drug that blocks acidification of the endosomal compartment (data not shown).

To ascertain that chloroquine is not affecting antigen presentation by altering MHC class II maturation, Sal/A^k/HEL cells were incubated in 0.5, 1, 2, 5, 10, or 20 μM chloroquine for 20 h and their MHC class II levels measured by flow cytometry following indirect immunofluorescence labeling with

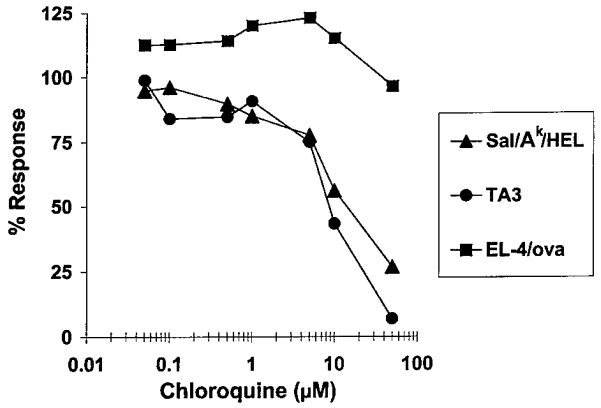

Figure 1: Chloroquine blocks MHC class II-restricted endogenous antigen presentation by Sal/A^k/HEL vaccines. Antigen-presenting cells, TA3 (●), EL-4/ova (■) or Sal/A^k/HEL (▲), were treated with chloroquine and incubated with 3A9 (TA3 and Sal/A^k/HEL) or B3Z (EL-4/ova) hybridoma cells. Antigen presentation was determined by comparing IL-2 release (TA3 and Sal/A^k/HEL) or β-gal activity (EL-4/ova) in the absence of chloroquine versus the presence of chloroquine. Average values of triplicate antigen presentation cultures without chloroquine were: TA3 + 3A9: 3876 pg/ml IL-2; EL-4/ova + B3Z: OD 415 nm 0.509; Sal/A^k/HEL + 3A9: 596.3 pg/ml IL-2. These data are representative of four independent experiments.

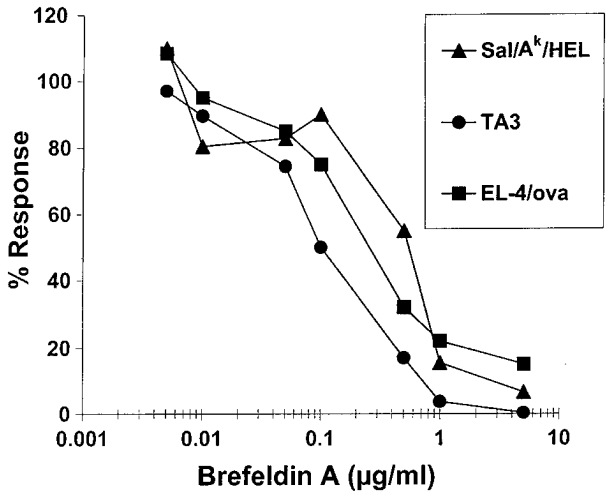

Figure 2: Brefeldin A blocks MHC class II-restricted endogenous antigen presentation by Sal/A^k/HEL vaccines. Antigen-presenting cells, TA3 (●), EL-4/ova (■), and Sal/A^k/HEL (▲), were treated with BFA and incubated with 3A9 (TA3 and Sal/A^k/HEL) or B3Z (EL-4/ova) hybridoma cells. Antigen presentation was determined by comparing IL-2 release (TA3 and Sal/A^k/HEL) or β-gal activity (EL-4/ova) in the absence of BFA versus the presence of BFA. Values without BFA were: TA3 + 3A9, 4425.1 pg/ml IL-2; EL-4/ova + B3Z, OD 0.55; Sal/A^k/HEL + 3A9: 727.6 pg/ml IL-2. These data are representative of four independent experiments.

mAb 10-2-16. Mean channel fluorescence for chloroquine-treated cells was 15.2, 14.7, 16.3, 15, and 16.6, respectively, versus 13.6 for untreated cells. Chloroquine, therefore, has no measurable effect on MHC class II maturation and expression, while it significantly alters endogenous antigen presentation. Presentation of class II-restricted endogenous antigen by the vaccines, therefore, involves the endocytic pathway.

To determine if MHC class II/endogenous antigen traffics via the Golgi, the drug BFA, which blocks transport of newly synthesized proteins from the endoplasmic reticulum (ER) to the Golgi, was used (26). Transit from the ER to the Golgi occurs in both the cytosolic and endocytic pathways. As shown in Figure 2, BFA efficiently blocks presentation of ovalbumin (ova) by the EL-4/ova cells (class I cytosolic pathway) and presentation of exogenous HEL by TA3 cells (class II endocytic pathway), and also blocks MHC class II-restricted endogenous HEL presentation by the Sal/A^k/HEL tumor vaccines. Egress from the ER to the Golgi, therefore, is required for presentation of endogenous, class II-restricted antigen by the tumor vaccines.

LLnL, but not lactacystin, E64 or leupeptin, block presentation

Antigen bound to MHC class II molecules consists of peptide fragments derived from larger protein antigens. Endogenously synthesized antigens trafficking via the cytosolic pathway and presented by MHC class I molecules are typically degraded into peptides by proteasomal proteases, while exogenously synthesized antigen presented by MHC class II

molecules is characteristically degraded into peptides in endosomal compartments (27,28). To ascertain that protease degradation is required for presentation of endogenously synthesized antigen by the tumor vaccines, antigen presentation assays were performed in the presence of LLnL. LLnL is a potent protease inhibitor that blocks protease activity of the proteasomes, endosomes, as well as other sites of cellular proteases (29,30). As shown in Figure 3, LLnL efficiently inhibits presentation via both the cytosolic (EL-4/ova APC) and endocytic (TA3 APC) routes, and equally inhibits antigen presentation by the tumor vaccines. Presentation of endogenously synthesized antigen by MHC class II molecules in the vaccines, therefore, requires protease-dependent degradation of endogenously encoded antigen, however, it is not clear from the LLnL experiments where degradation occurs.

To help localize the intracellular site of proteolysis for endogenously synthesized antigen, the proteasome inhibitor lactacystin (31,32) was used. Lactacystin specifically blocks degradation of proteins by the proteasome without inhibiting endosomal/lysosomal degradation or having other effects on the endocytic class II pathway (33). As shown in Figure 4, lactacystin efficiently blocks presentation of ova by the EL-4/ova cells (cytosolic pathway), but does not inhibit presentation of exogenous HEL by TA3 cells (endocytic pathway) or endogenous HEL by Sal/A^k/HEL vaccines. Proteasomal degradation of HEL, therefore, is not required for antigen presentation by the tumor vaccines.

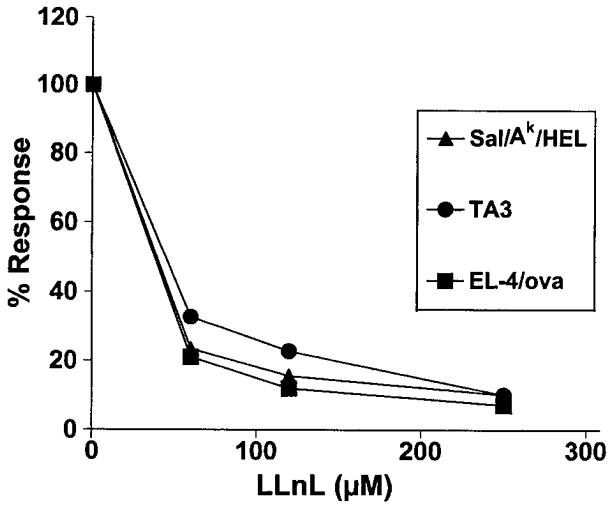

Figure 3: LLnL inhibits MHC class II-restricted endogenous antigen presentation by Sal/A^k/HEL vaccines. Antigen-presenting cells, TA3 (●), EL-4/ova (■), and Sal/A^k/HEL (▲), were treated with LLnL and incubated with 3A9 (TA3 and Sal/A^k/HEL) or B3Z (EL-4/ova) hybridoma cells in the presence of the indicated concentrations of LLnL. Antigen presentation was determined by comparing IL-2 release (TA3 and Sal/A^k/HEL) or β-gal activity (EL-4/ova) in the absence of LLnL vs. the presence of LLnL. Values without LLnL were: TA3 + 3A9: 3579.4 pg/ml IL-2; EL-4/ova + B3Z: OD 0.84; Sal/A^k/HEL + 3A9: 356.6 pg/ml IL-2. The data are representative of two independent experiments.

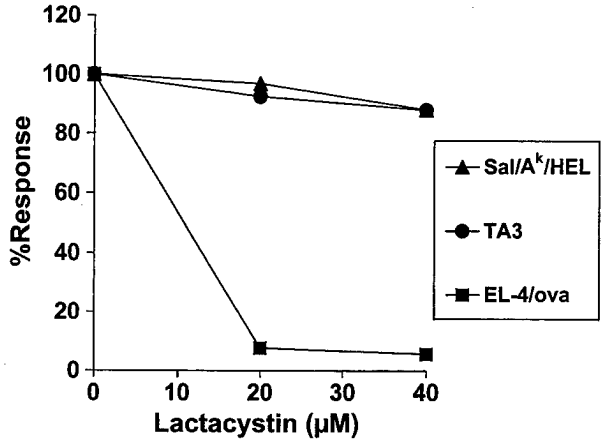

Figure 4: Lactacystin does not block MHC class II-restricted endogenous antigen presentation by Sal/A^k/HEL vaccines. Antigen-presenting cells, TA3 (●), EL-4/ova (■), and Sal/A^k/HEL (▲), were treated with lactacystin and incubated with 3A9 (TA3 and Sal/A^k/HEL) or B3Z (EL-4/ova) hybridoma cells. Antigen presentation was determined by comparing IL-2 release (TA3 and Sal/A^k/HEL) or β-gal activity (EL-4/ova) in the absence of lactacystin versus the presence of lactacystin. Values without lactacystin were: TA3 + 3A9: 3425.6 pg/ml IL-2; EL-4/ova + B3Z: OD 0.69; Sal/A^k/HEL + 3A9: 453.1 pg/ml IL-2. These data are representative of three independent experiments.

To determine if HEL proteolysis occurs in the endosomal compartment, antigen presentation assays were performed in the presence of E64 (34), an inhibitor of cysteine proteases such as papain and the endosomal cathepsins B and L, and leupeptin (30), an inhibitor of serine and cysteine proteases such as trypsin, papain, and the endosomal protease cathepsin B. As shown in Figure 5, E64 (panel A) and leupeptin (panel B) do not inhibit endogenous HEL presentation by Sal/A^k/HEL cells. In contrast, E64 and leupeptin inhibit presentation of exogenous intact HEL by Sal cells transduced with the MHC class II transactivator gene (Sal/CIITA cells). Sal/CIITA cells have previously been shown to be efficient presenters of exogenous antigen (16). As expected, the drugs do not inhibit presentation of exogenous HEL peptide 46-61 by Sal/CIITA cells. Pepstatin A, an inhibitor of aspartic acid proteases such as pepsin and the endosomal aspartic acid protease cathepsin D, was also tested for its ability to block endogenous HEL presentation. Antigen presentation assays were performed in the presence of pepstatin A, however, even at concentrations as high as 50 μg/ml, presentation of exogenous intact HEL and endogenous HEL was not blocked (data not shown). HEL proteolysis, therefore, does not occur in the endosomal compartment, despite trafficking of MHC class II molecules via this route.

Collectively, the drug experiments indicate that class II-restricted endogenous antigen presentation by the tumor vaccines occurs via the endocytic pathway, even in the absence of Ii chain, but HEL degradation does not occur in the endosome, and components of the cytosolic pathway, such as the proteasome, are not involved.

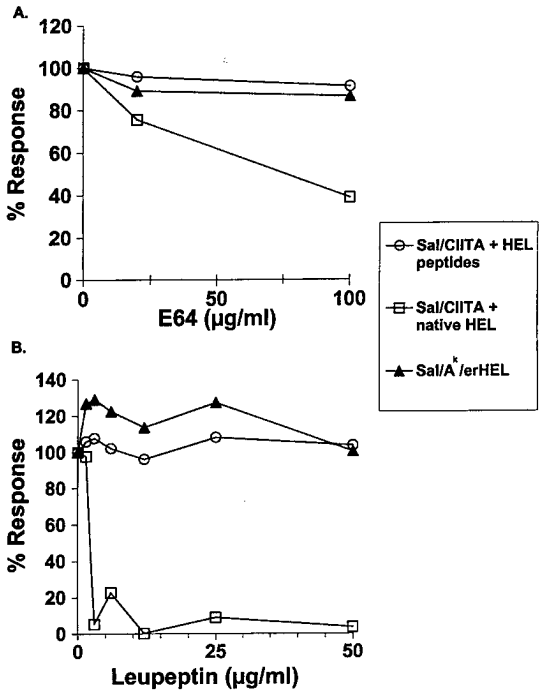

Figure 5: Protease inhibitors E64 and leupeptin do not inhibit presentation of endogenous antigen by the vaccines. Sal/A^k/HEL (▲), Sal/CIITA + HEL protein (□), or Sal/CIITA + HEL peptide (○) were cultured with E64 (panel A) or leupeptin (panel B) for 16 h, fixed with paraformaldehyde, and then incubated with 3A9 hybridoma cells. Supernatants were harvested and assayed by ELISA for IL-2 activity. These data are representative of two independent experiments.

Transfection with the H-2Ma and H-2Mb genes

H-2M is a critical molecule for optimal loading of exogenously synthesized antigen onto MHC class II molecules in the endocytic pathway. Because the endocytic pathway appears to play a role in presentation of endogenously synthesized tumor antigens by class II molecules, it is possible that H-2M also affects loading of endogenously synthesized antigen onto class II molecules. To test this hypothesis, Sal/A^k and Sal/A^k/HEL tumor vaccines were transfected with plasmids containing the *H-2Ma* and *H-2Mb* genes. Figure 6 shows the flow cytometry profiles of two Sal/A^k/DM/HEL clones, two Sal/A^k/DM clones, and control non-H-2M-expressing Sal, Sal/A^k, Sal/A^k/HEL, Sal/A^k/Ii, and Sal/A^k/Ii/HEL cells stained for MHC class II (I-A^k), H-2M (DM), Ii, and HEL. Sal cells transduced with the class II transactivator gene (Sal/CIITA), which coordinately up-regulates MHC class II, Ii, and H-2M genes (35,36), are also shown. Panels s-x of Figure 6 show the HEL staining profiles for non-HEL-transfected cells, and panels y-cc show the profiles for the HEL transfectants. For all transfectants, levels of the transfected genes are approximately equal among the transfectants.

H-2M expression enhances exogenous presentation

To confirm that the H-2M αβ dimer is functional in vivo and that H-2M expression enhances presentation of exogenous, class II-restricted antigen, we have used the 3A9 hybridoma and the RNase (42–56)-specific, I-A^k-restricted, CD4+ TS12 hybridoma. As shown in Figure 7, H-2M transfectants (Sal/A^k/DM1 and Sal/A^k/DM2) efficiently present exogenous HEL (Figure 7A) and RNase (Figure 7B), while Sal/A^k and Sal/A^k/Ii transfectants not expressing H-2M do not present exogenous antigen. Interestingly, the vaccines that express class II

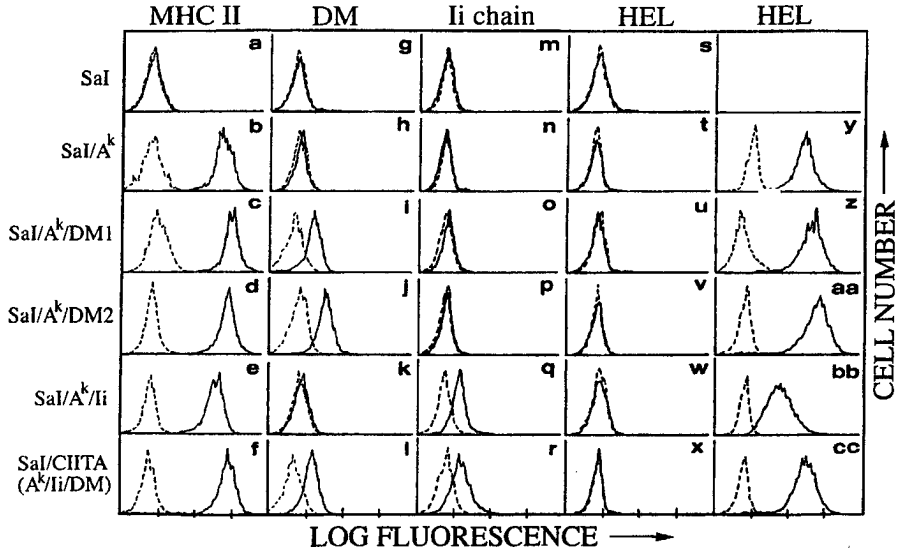

Figure 6: MHC class II (I-A^k), H-2M, Ii chain, and HEL expression in Sal and Sal transfectants. Sal (panels a,g,m,s), Sal/A^k (panels b,h,n,t), Sal/A^k/DM1 (panels c,i.o,u), Sal/A^k/DM2 (d,j,p,v), Sal/A^k/Ii (panels e,k,q,w), and Sal/CIITA (panels f,l,r,x) were stained with antibodies to I-A^k (mAb 10-2.16, panels a-f), H-2M (polyclonal antibody K553, panels g-l), Ii (mAb In-1, panels m-r), or HEL (mAbs HyHEL 7 and 10, panels s-cc). HEL transfectants Sal/A^k/HEL, Sal/A^k/DM1/HEL, Sal/A^k/DM2/HEL, Sal/A^k/Ii/HEL, and Sal/CIITA/HEL were stained with mAbs HyHEL 7 and 10 (panels y-cc, respectively).

and H-2M without Ii present exogenous antigen more efficiently than vaccines that express class II, Ii, and H-2M (Sal/A^k/DM vs. Sal/CIITA). As expected, co-expression of H-2M, therefore, facilitates presentation of exogenous antigen by class II molecules.

H-2M expression does not affect endogenous presentation

The role of H-2M expression in presentation of endogenous antigen via the endocytic pathway was tested using the 3A9 hybridoma and the various Sal/A^k vaccines expressing endogenously encoded HEL as APC. As shown in Figure 8, Sal/A^k/HEL and the two Sal/A^k/DM/HEL vaccines (Sal/A^k/DM/HEL.1, Sal/A^k/DM/HEL.2) stimulate approximately equivalent levels of IL-2 release. If the vaccines co-express Ii, they do not present endogenous antigen (Sal/A^k/Ii/HEL), even if H-2M is also expressed (Sal/CIITA/HEL). H-2M expression, therefore, does not affect presentation of endogenously synthesized antigen by class II molecules.

Tumorigenicity is not affected by H-2M expression

In previous studies vaccine efficacy has been directly proportional to tumorigenicity of the unirradiated vaccine (5,16,36). Therefore, immunocompetent, tumor-free syngeneic A/J mice were challenged with the various Sal/A^k vaccines (transfectants) to ascertain if H-2M expression affects tumor growth. In parallel, irradiated A/J mice were also challenged with the vaccines to ascertain that the transfectants have not lost their inherent malignant potential. As shown in Table 1, transfectants that express MHC class II, with or without H-2M, are rejected by immunocompetent A/J mice, while transfectants co-expressing Ii (Sal/A^k/Ii and Sal/CIITA) are lethal. All vaccines are lethal in immunocompromised, irradiated mice. Tumor rejection, therefore, correlates with the presence of MHC class II, is not affected by H-2M expression, and is inversely correlated with Ii expression by the vaccine (summarized in Table 2).

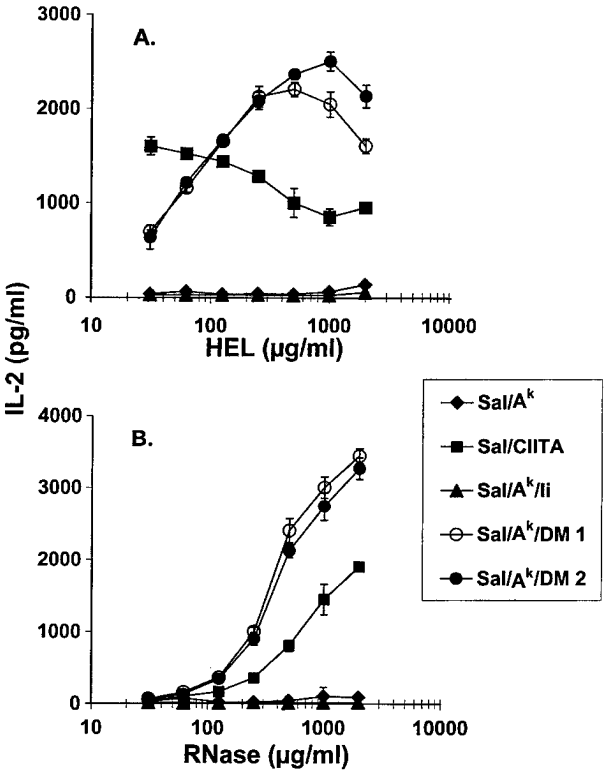

Figure 7: Expression of H-2M in Sal/A^k cells (Sal/A^k/DM transfectants) enables presentation of exogenously synthesized HEL and RNase. Sal/A^k (◆), Sal/A^k/DM1 (○), Sal/A^k/DM2 (●), Sal/CIITA (■), or Sal/A^k/Ii (▲) cells were cocultured with 3A9 (A) or TS12 (B) hybridoma cells in the presence of varying doses of exogenous HEL (A) or RNase (B). These data are representative of seven independent experiments.

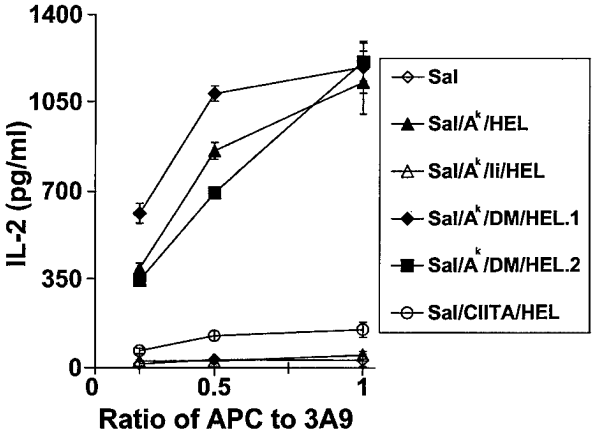

Figure 8: Expression of H-2M in Ii negative Sal/A^k cells does not affect presentation of endogenously synthesized ER-localized HEL. Tumor vaccine cells (APC; Sal ◇, Sal/A^k/HEL ▲, Sal/A^k/Ii/HEL △, Sal/A^k/DM/HEL.1 ◆, Sal/A^k/DM/HEL.2 ■, Sal/CIITA/HEL ○) were incubated with 3A9 T hybridoma cells, and IL-2 release measured by ELISA. These data are representative of four independent experiments.

Discussion

In addition to presenting exogenously synthesized MHC class II-restricted antigens, professional APC also present class

Table 1: Co-expression of H-2M does not change the immunogenicity of MHC class II-transfected sarcoma tumor cells

Tumor cells	Tumor incidence in	
	Irradiated mice	Non-irradiated mice
Sal		5/5
Sal/A^k	3/3	0/10
Sal/A^k/Ii	2/2	6/8
Sal/A^k/DM 1	3/4	0/11
Sal/A^k/DM 2	2/2	0/11
Sal/CIITA (Sal/A^k/Ii/H-2M)	2/2	5/5

Immunocompetent or irradiated syngeneic A/J mice were challenged i.p. with 10^6 of the indicated tumor cells. Mice were followed for tumor growth. Tumor incidence is the number of mice with tumors divided by the total number of mice challenged with tumor.

Table 2: Summary table of tumorigenicity and antigen presentation activity of the Sal/A^k tumor vaccines

Vaccine	In vitro antigen presentation of		Tumorigenicity	Reference
	Endogenous antigen	Exogenous antigen		
Sal	−−	−−	++	(16,37)
Sal/A^k	++	−−	−−	(5,17)
Sal/A^k/Ii	−−	−−	++	(16,37)
Sal/A^k/DM	++	++/+++	−−	This report
Sal/CIITA	−−	++	++	(16)

'−−' indicates no antigen presentation or no tumor incidence; '++' indicates moderate antigen presentation or 75% tumorigenicity; '+++' indicates very strong antigen presentation.

II-restricted endogenously synthesized antigens on class II MHC molecules (19). We have exploited this capability and generated MHC class II^+Ii^- tumor vaccines that directly activate $CD4^+$ MHC class II-restricted tumor-specific lymphocytes and are therapeutically useful for the treatment of established metastatic cancer ((11), B. Pulaski and S. Ostrand-Rosenberg, submitted). Although other investigators (20,21) have studied presentation of endogenously encoded peptides, in these studies, soluble peptides were fed to APC so that the peptides were effectively delivered via an exogenous route, rather than being naturally processed via an endogenous route within the APC. In addition, the APC used in these studies were professional APC that expressed invariant chain and other class II-associated accessory molecules. In contrast, the vaccine cells used here do not contain additional class II-associated accessory molecules and present antigen that is encoded and processed within the vaccine cells. To clarify potential differences between antigen presentation by the vaccines and by professional APC, we have, therefore, studied MHC class II antigen trafficking pathways in the vaccines, and anticipate that an understanding of this process may lead to improved vaccine therapeutic efficacy.

Antigen presentation involves at least three distinct steps: 1) degradation of antigen into peptide; 2) loading of peptide onto class II; and 3) trafficking of the class II/peptide complexes to the cell surface. Although our experiments do not precisely distinguish where HEL degradation occurs, we can draw several conclusions from our results. Because lactacystin, E64, and leupeptin do not inhibit antigen presentation, it is unlikely that HEL degradation occurs by proteases of the proteasome or a late endosomal compartment. Early studies demonstrated that protein is possibly degraded in the ER (38–40), however, more recent reports suggest that proteolysis is relatively inefficient in the ER (41,42). Alternatively, degradation may occur in the recently described alternative cytosolic proteolytic system (43–45). If the cytosol is the site of degradation, then ER-tethered HEL must exit the ER, be degraded in the cytosol, and be transported back into the ER where it would bind to newly synthesized class II molecules. Transport from the cytoplasm into the ER could be mediated by a transporter such as TAP. This hypothesis could be tested using inhibitors of TAP.

Based on our results, we can eliminate certain sites for peptide binding to class II molecules. Peptide loading onto class II molecules is very inefficient in the ER (41,42) and, hence, binding here is unlikely. It is also improbable that class II molecules bind peptides in the late endosome (MIIC), because Sal/CIITA/HEL vaccines (Ii^+DM^+) do not present endogenously synthesized HEL, while they efficiently present exogenous HEL which is bound in the late endosome. However, peptide binding could occur in the early endosome where exogenous peptide loading is very inefficient (46). Peptide binding in the early endosome would stabilize newly synthesized empty class II molecules and would be independent of H-2M in the vaccines, because in the absence of Ii, H-2M would not be required to displace CLIP (class II peptide). This model is supported by the finding that H-2M expression does not affect binding of endogenously synthesized peptides in Sal/A^k cells. Although peptide binding could occur in the early endosome in the vaccines, it is unlikely that peptide would bind in the early endosome in professional APC, because class II molecules in professional APC would be occupied by Ii, and H-2M would not be present to facilitate specific peptide binding. Therefore, if class II binds peptide in the early endosome, presentation of endogenously synthesized class II-restricted antigen, may differ between professional APC and the tumor vaccines described here.

An acidified endosomal compartment is clearly necessary for presentation of an ER-localized antigen by the vaccines, because chloroquine treatment inhibits antigen presentation activity. In professional APC, MHC class II molecules are guided to the endosome by trafficking signals contained within the Ii chain. The tumor vaccines, however, do not contain Ii. Presumably in the absence of Ii, directional signals imbedded in the class II β chain are sufficient to direct the complexes to the endosome (47–49).

Results presented here confirm that H-2M profoundly affects presentation of exogenous HEL (50,51), but contradict other studies suggesting that RNase presentation is H-2M inde-

pendent (52). This discrepancy may be due to the APC rather than the antigen. In the latter study, T2 cells, a T/B hybrid line, were used. Because these cells are derived from professional APC they may express additional molecules that affect antigen presentation. Presentation of specific antigens, therefore, may not be exclusively dependent on the individual antigen, but also regulated by the environment of the APC.

In contrast to presentation of exogenous antigen, H-2M does not influence endogenous antigen presentation by the vaccines. This result is surprising because, during presentation of exogenous antigen, H-2M continually edits not only CLIP, but other peptides as well (53). It is not obvious why H-2M should not perform this same editing function to optimize antigen presentation of endogenously encoded antigens.

Molecules/peptides defined as tumor antigens have been identified in the cytosol, nucleus, plasma membrane, and as secreted products of tumor cells (1,2). An effective tumor cell-based cancer vaccine, therefore, must present peptides from all of these subcellular compartments. In addition to the ER-retained endogenous antigen of this report, the vaccines also present membrane-expressed endogenously synthesized molecules (16) and molecules targeted to the cytoplasm and nucleus (L. Qi, J. Rojas, and S. Ostrand-Rosenberg, unpublished results). Because tumor antigens have been identified in all of these subcellular compartments, the tumor vaccines may be very useful reagents for inducing immunity to a wide spectrum of potential rejection antigens. As demonstrated by others, MHC class II molecules of Ii^-DM^- APC present a different repertoire of peptides than class II molecules of Ii^+DM^+ APC (54). The therapeutic efficacy of the vaccines, therefore, may be the result of their ability to present novel tumor peptides and therefore stimulate tumor-specific $CD4^+$ T cells that would not be induced by conventional, professional Ii^+DM^+ APC.

Materials and Methods

Mice and tumor challenges

Five- to 8-week-old A/J (*H-2K^{k}A^{k}E^{k}D^d*) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred and maintained in the UMBC animal facility according to NIH guidelines for humane treatment of laboratory animals. Mice were inoculated intraperitoneally (i.p.) with 10^6 tumor cells and followed for tumor incidence as previously described (5). Irradiated mice received 875 rads total body irradiation (Gammator B, Cs-137 source, Kewaunee Scientific, Statesville, NC).

Cell lines and transfectants

Sal is an A/J-derived mouse sarcoma. Sal/A^k, Sal/A^k/Ii, Sal/CIITA, Sal/A^k/HEL, Sal/A^k/Ii/HEL, and Sal/CIITA/HEL were generated from Sal cells by transfection with plasmids containing *I-Aa^k* and *I-Ab^k* MHC class II cDNA, *I-A^k* plus *Ii*, and class II transactivator (CIITA) genes, respectively, with or without the ER-retained hen eggwhite lysozyme gene (HEL; HEL containing the KDEL ER retention signal) as previously described (5,16,37). H-2M expressing tumor lines were generated by stable transfection (16). Briefly, 10^6 Sal/A^k and Sal/A^k/HEL cells were plated in serum free medium (Optimem, GIBCO/BRL, Grand Island, NY) in 6-cm petri dishes, cocultured with 4 μg each *H-2Ma* and *H-2Mb* plasmids and 2 μg of the pZeoSV2 plasmid (Invitrogen) encoding zeocin resistance for 16 h with 20 μl lipofectin (GIBCO/BRL, Carlsbad, CA). After a 24-h incubation period in culture medium, selection was applied by adding 200 μg/ml zeocin (Invitrogen). Transfectants were cloned by limiting dilution, tested for H-2M expression by indirect immunofluorescence, and two clones of each line (Sal/A^k/DM1, Sal/A^k/DM2, Sal/A^k/DM1/HEL, Sal/A^k/DM2/HEL) selected for further study. H-2M transfectants were maintained in culture medium supplemented with 400 μg/ml G418, 400 μg/ml hygromycin and 200 μg/ml zeocin and were tested approximately once a month for H-2M expression.

TA3 is an I-A^k-expressing B cell lymphoma. 3A9 is a HEL (46–61)-specific I-A^k-restricted T cell hybridoma and is maintained in IMDM supplemented with 10% FBP (55). TS12 is a RNase (42–56)-specific I-A^k-restricted T cell hybridoma and is maintained in RPMI1640 (GIBCO), 10%FCS, Glutamax II and 1% Gentamicin (56). EL-4/ova cells present the ovalbumin peptide 256–264 bound to H-2K^b (57). B3Z is an ova (256-264)-specific, H-2K^b-restricted, $CD8^+$ T cell hybridoma fused to a LacZ-inducible T cell line. Activation of B3Z is measured by β-galactosidase (β-gal) production (58). EL-4/ova and B3Z cells are maintained in RPMI1640, supplemented with10%FCS, 400 μg/ml G418, 2 mM Glutamax I, and 10^{-5} M β-ME.

Plasmids and antibodies

Mouse *H-2Ma* and *H-2Mb* cDNAs are from (59). MHC class II-specific mouse mAb 10-2-16 (60), rabbit polyclonal antibody K553 to H-2M β chain (59), rat mAb In-1 to Ii chain (36), and mouse mAbs hyHEL 7 and 10 to HEL (61) were previously described.

Indirect immunofluorescence and flow cytometry

Tumor cells were stained by indirect immunofluorescence either externally or internally and analyzed on an Epics XL flow cytometer as previously described (16). The histograms were analyzed using the XL program and/or WinMDI software (http://facs.scripps.edu/).

Antigen presentation assays

HEL and RNase antigen presentation assays were performed as previously described for HEL (16) with minor modifications. Briefly, for endogenous antigen presentation assays, APCs (Sal, Sal/A^k/HEL, Sal/A^k/Ii/HEL, Sal/A^k/DM/HEL, or Sal/CIITA/HEL cells) were cocultured overnight (12–16 h) with 5×10^4 3A9 hybridoma cells. For exogenous antigen presentation assays HEL or RNase, at the indicated concentrations, was added to the wells containing non-HEL-transfected APC (Sal/A^k, Sal/A^k/Ii, Sal/A^k/DM, or Sal/CIITA) plus 3A9 or TS12 hybridoma cells, respectively.

Antigen presentation assays with drugs

Working stocks of chloroquine (100 mM), lactacystin (5 mM), LLnL (20 mM), leupeptin (1 mg/ml) in H_2O, BFA (1 mg/ml) in ethanol, and E64 (1 mg/ml) in 1:1 water:ethanol were used.

For exogenous antigen presentation assays using TA3 or Sal/CIITA APC, 1×10^6 cells in 2 ml culture medium were incubated with drugs at the indicated concentrations for 30 min. A total of 500 μg/ml native HEL or HEL peptide (46–61) was then added and the cells were incubated for 16 h at 37°C. The cells were then washed once with excess cold PBS, fixed with 1% cold paraformaldehyde for 15 min, and washed twice with cold culture medium. Then, 1×10^5 APC cells were mixed with 1×10^5 hybridoma cells in a total volume of 200 μl per well in 96-well flat-bottomed plates. Drug-treated TA3 cells and

responding 3A9 cells were then incubated for 6 h at 37°C, and supernatants removed and tested for IL-2 content by ELISA.

Endogenous antigen presentation assays using Sal/A^k/HEL cells and inhibitory drugs were performed as described above for exogenous APC assays with drugs, except exogenous antigen was not added.

For EL-4/ova experiments, 5×10^6 APC in 2 ml culture medium were washed once with PBS, followed by a wash with mild acid buffer (131 mM citric acid and 66 mM disodium phosphate, pH 3.1) at 25°C for 3 min (62). Cells were then neutralized by addition of a 30-fold excess of culture medium. Acid-washed EL-4/ova were either fixed with 1% paraformaldehyde (15 min on ice; control APC), or incubated at 37°C for 16 h with drug. Following drug-treatment, EL-4/ova cells were fixed with 1% paraformaldehyde, and then washed twice with excess cold culture medium. Drug-treated EL-4/ova cells were then incubated at 37°C for 6 h with 1×10^5 B3Z hybridoma cells. The cultures were then washed once with 100 µl PBS, and cells lysed by addition of 100 µl per well of 10 mM ONPG in PBS/0.5% NP-40. Following a 4-h incubation at 37°C, β-galactosidase activity in the supernatants was measured using a plate reader equipped with a 415 nm filter (63).

For IL-2 assays with TA3 and Sal/A^k/HEL, % Response = 100% × [IL-2 (with drug)/IL-2 (without drug)]; for LacZ assay of EL-4/ova, % Response = 100% × ([OD 415 nm (with drug) − OD415 nm (acid wash)]/[OD 415 nm (without drug) − [OD 415 nm (acid wash)]). All points were run in triplicate and the means and standard deviations for each experimental condition were determined. Standard deviations ranged from 0.5–6%.

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ORIGINAL ARTICLE

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Immunotherapy with vaccines combining MHC class II/CD80$^+$ tumor cells with interleukin-12 reduces established metastatic disease and stimulates immune effectors and monokine induced by interferon γ

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Abstract Because they are difficult to treat, animal models of widespread, established metastatic cancer are rarely used to test novel immunotherapies. Two such mouse models are used in this report to demonstrate the therapeutic efficacy and to probe the mechanisms of a novel combination immunotherapy consisting of the cytokine interleukin-12 (IL-12) combined with a previously described vaccine based on MHC class II, CD80-expressing cells. BALB/c mice with 3-week established primary 4T1 mammary carcinomas up to 6 mm in diameter and with extensive, spontaneous lung metastases show a significant reduction in lung metastases following a 3-week course of immunotherapy consisting of weekly injections of the cell-based vaccine plus injections of IL-12 three times per week. C57BL/6 mice with 7-day established intravenous B16 melF10 lung metastases show a similar response following immunotherapy with IL-12 plus a vaccine based on B16 MHC class II, CD80-expressing cells. In both systems the combination therapy of cells plus IL-12 is more effective than IL-12 or the cellular vaccine alone, although, in the 4T1 system, optimal activity does not require MHC class II and CD80 expression in the vaccine cells. The cell-based vaccines were originally designed to activate tumor-specific CD4$^+$ T lymphocytes specifically and thereby provide helper activity to tumor-cytotoxic CD8$^+$ T cells, and IL-12 was added to the therapy to facilitate T helper type 1 lymphocyte (Th1) differentiation. In vivo depletion experiments for CD4$^+$ and CD8$^+$ T cells and natural killer (NK) cells and tumor challenge experiments in beige/nude/XID immunodeficient mice demonstrate that the therapeutic effect is not exclusively dependent on a single cell population, suggesting that T and NK cells are acting together to optimize the response. IL-12 may also be enhancing the immunotherapy via induction of the chemokine Mig (monokine induced by interferon γ), because reverse PCR experiments demonstrate that Mig is present in the lungs of mice receiving therapy and is most likely synthesized by the tumor cells. These results demonstrate that the combination therapy of systemic IL-12 and a cell-based vaccine is an effective agent for the treatment of advanced, disseminated metastatic cancers in experimental mouse models and that multiple effector cell populations and anti-angiostatic factors are likely to mediate the effect.

Key words Immunotherapy · Metastatic mammary carcinoma · IL-12 · Angiogenesis · CD80 · MHC class II · CD4$^+$ T cells

Introduction

Many of the recently explored immunotherapy strategies for the treatment of cancer have focused on the improved activation of tumor-specific immunity. For example, administration of interleukin-12 (IL-12), a cytokine that favors T helper type 1 lymphocytes (Th1) and natural killer (NK) cell development and stimulates anti-angiogenic chemokines [12, 20], reduces tumor burden in numerous mouse tumor systems [4, 38, 39]. Likewise, the treatment of mice with established primary and/or metastatic tumor with irradiated immunogenic tumor cells, constitutively expressing MHC class I molecules and transfected/transduced with the costimulatory molecule CD80, reduces primary tumor mass and/or small metastatic tumor load [6, 41]. This latter approach is based on the premise that the genetically engineered tumor cells present both antigen-specific and costimulatory T cell activation signals to the relevant CD8$^+$ T lymphocytes.

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Another immunotherapeutic strategy aimed at specifically improving the generation of tumor-specific $CD4^+$ T lymphocytes uses autologous tumor cells transfected with syngeneic MHC class II genes plus CD80 costimulatory molecule genes as cell-based vaccines for the treatment of mice with established primary and metastatic cancer. This therapy is based on the hypothesis that enhanced generation of $CD4^+$ tumor-specific T helper lymphocytes facilitates $CD8^+$ T cell activation and promotes stable, long-term immune memory against recurrence of primary tumor and/or outgrowth of micrometastases [27, 28]. Treatment with MHC-class-II-transfected-cell-based vaccines has yielded significant reductions in solid tumor mass [1, 2] and in established, spontaneous metastatic disease [32].

In an attempt to generate a more potent antitumor effect, IL-12 and CD80 therapies have been combined to target the activation of $CD8^+$ T cells. Because in vitro studies have shown that IL-12 plus CD80 produces optimal T cell proliferation and interferon γ (IFNγ) production [23, 25] as well as stimulating a primary antitumor response in vitro [17], it is not surprising that IL-12 and CD80 synergize to bring about significant regression of established primary tumor as well as inducing immunological memory against recurrence of primary tumor [11, 33]. Although a principal function of IL-12 is its ability to promote $CD4^+$ Th1 differentiation, surprisingly, IL-12 therapy has not previously been combined with other therapies that specifically target the activation of tumor-specific $CD4^+$ T cells. To test the potential effect of targeting with IL-12 plus $CD4^+$, we have combined systemic IL-12 therapy with immunization using MHC class II/CD80 genetically modified tumor cells for the treatment of established (induced i.v. and spontaneous) metastatic disease. Previous in vivo studies testing IL-12 therapy have used mouse tumor models consisting of either solid, subcutaneous primary tumors, or very early metastases induced by intravenous injection of malignant cells [4, 7, 9, 10, 11, 31, 33]. Although these model systems provide some insight into the potential role of therapeutic agents in the treatment of cancer, they are not realistic clinical situations in which larger metastatic tumor loads are likely to be encountered and for which more effective treatments are needed.

To test potential immunotherapies more rigorously against larger metastatic loads, we have used two mouse tumor systems. The 4T1 mammary carcinoma tumor is a very poorly immunogenic and highly malignant tumor that rapidly and spontaneously metastasizes to lymph nodes, lung, liver, brain, and blood following growth of the primary tumor in the mammary gland [24, 32]. This disease progression closely parallels human breast cancer and makes the 4T1 tumor an excellent model for human disease [32] and a rigorous animal model of advanced spontaneous metastatic disease.

As a second model system, we have used the B16-derived melF10 melanoma [16]. This tumor is also very poorly immunogenic and highly malignant, and metastasizes immediately to the lung when inoculated intravenously. Similar to approximately 15% of human cancers, melF10 has markedly reduced levels of MHC class I molecules. This phenotype probably contributes to its reduced immunogenicity and heightened tumorigenicity. Many investigators have used the melF10 tumor as an experimental model; however, most studies use lung metastases very early after they are established (e.g. 3 days or less after i.v. inoculation). We have used longer-established melF10 lung metastases (therapy begins on day 7 after i.v. inoculation) to test the combination vaccine more rigorously. For both tumors, the combined therapy is more effective than either therapy alone, and appears to be mediated by multiple independent effector mechanisms including T lymphocytes, NK cells, and possibly chemokine production that has been linked to anti-angiogenesis.

Materials and methods

Cells and transfectants

MelF10 is a high metastatic variant of the C57BL/6-derived B16 melanoma [16]. 4T1 is a spontaneously metastatic, poorly immunogenic BALB/c-derived mammary carcinoma [24]. Culture conditions for both tumors have been previously described [29, 32]. Generation and characterization of 4T1 transfectants expressing $I\text{-}A^d$ and CD80 and B16melF10 transfectants expressing $I\text{-}A^b$ and CD80 have been previously described [29, 32].

Mice Mice were purchased from The Jackson Laboratory (Bar Harbor, Me.) or bred in the UMBC Animal Facility from breeding pairs purchased from The Jackson Laboratory. Experiments using the 4T1 mammary carcinoma or melF10 melanoma were performed in female BALB/c mice and C57BL/6 male or female mice, respectively. All mice were between 6 weeks and 6 months in age.

Tumor challenges and metastases assays

Tumorigenesis and metastasis formation by the melF10 [29] and 4T1 [32] tumors were performed as previously described. Briefly, for experimental metastases, 10^5 melF10 cells/100 µl for each mouse were inoculated intravenously (i.v.) into the tail vein of C57BL/6 mice on day 0; the mice were sacrificed 3–4 weeks later and their lungs observed and weighed. For spontaneous metastases 7×10^3 4T1 cells/50 µl for each mouse were inoculated into the abdominal mammary gland of BALB/c female mice on day 0; the mice were sacrificed 6 weeks later and the number of clonogenic metastatic cells in the lungs assessed by growth in medium supplemented with 6-thioguanine [32]. Mice carrying tumors were closely followed for symptoms of pain and distress and were sacrificed when they became moribund. On the basis of previous studies [32], 4T1-bearing mice with up to 10 000 clonogenic metastatic 4T1 cells in their lungs are considered responder mice. All animal procedures followed the *Principles of laboratory animal care* (NIH publication 85-23, revised 1985) and were approved by the UMBC Institutional Animal Care and Use Committee.

Antibodies

Protein A or protein G purification of MHC-class-II-specific mAb 3JP ($I\text{-}A^b$ [19]), MKD6 ($I\text{-}A^d$ [21]), MHC-class-I-specific mAb 20-8-4 ($H\text{-}2\ K^b$ [30]), 28-14-8 ($H\text{-}2D^b$ [30]), CD4 (GK1.5 [42]), and CD8 (2.43 [35]) was as previously described [29, 32]. Fluorescently coupled CD3, CD4, CD8, NK1.1, and B220 mAb were purchased from Pharmingen.

In vivo depletions for CD4$^+$ and CD8$^+$ T cells and NK cells

Depletions of CD4$^+$ and CD8$^+$ T cells were performed as previously described [2]. Briefly, mAb to CD4$^+$ (GK1.5) and CD8$^+$ (2.43) T lymphocytes were prepared as ascites fluid in nude mice and had titers of at least 1/80 000 as measured by immunofluorescence. Mice were inoculated intraperitoneally (i.p.) with 100 µl (GK1.5) or 150 µl (2.43) ascites fluid mixed with an equal volume of phosphate-buffered saline on days −6, −3, and −1 before tumor challenge, and then once a week during tumor growth. Depletion resulted in elimination of 90%–100% of splenic CD4$^+$ T cells and 100% of splenic CD8$^+$ T cells as measured by immunofluorescence at the conclusion of the experiment.

BALB/c mice were depleted for NK cells by i.p. inoculation with 40 µl anti-asialoGM1 antiserum (Wako Pharmaceuticals; reconstituted as directed by manufacturer) on days −4 and −1 before therapy began and twice a week while therapy continued until the day of sacrifice. To ascertain NK depletions, anti-asialoGM1-treated mice and control rabbit-Ig-treated mice were boosted with 1 mg poly(I,C) i.p at the conclusion of the experiment and, 24 h later, their spleens were tested for NK activity by ^{51}Cr-release assay. At E:T ratios of 100:1, 50:1, and 25:1, anti-asialoGM1-depleted mice yielded 12%, 1%, and 0% cytotoxicity, respectively, compared to control rabbit-antibody-treated mice, which yielded 22%, 8%, and 6% cytotoxicity, respectively, against YAC-1 targets.

NK assays

NK assays were performed as previously described [26] using P815 cells as NK-resistant targets and YAC-1 cells as NK-susceptible targets. Briefly, target cells (up to 10^7) were radiolabeled with ^{51}Cr for 1.5 h and chased for 30 min at 37 °C in a volume of 0.5 ml. Labeled targets (5×10^4/well) were incubated with splenocytes (effectors) at ratios ranging from 100:1 to 6.25:1 in a final volume of 200 µl/well in 96-well plates. Cells were incubated at 37 °C for approximately 4 h, and supernatants harvested and counted in a Wallac minigamma counter. The percentage chromium release was calculated as [experimental release (cpm) − spontaneous release (cpm)]/[total release (cpm) − spontaneous release (cpm)] × 100. Mice were induced for NK activity by i.p. inoculation of 100 µl poly(I,C) 24 h prior to removal of spleens.

IL-12 and cell therapy

IL-12 therapy consisted of i.p. inoculations of 1 µg/mouse three times per week for 3 weeks. This dose was based on previous studies [39]. Cell therapy consisted of i.p. inoculations of 10^6 irradiated (50 Gy) melF10, 4T1, melF10 transfectants, or 4T1 transfectants once a week for 3 weeks. Therapy for 4T1 and melF10 experiments was started 21 and 7 days after wild-type tumor challenge respectively.

In vitro and in vivo detection of monokine induced by IFNγ (Mig) by reverse polymerase chain reaction (PCR)

4T1 and MelF10 cell lines were induced in vitro with 100 U/ml interferon γ (IFN-γ) for 2 h. Naive mice were injected with 4T1 parental tumor and treated with immunotherapy as described above. Lung tissue was removed at the indicated times after the start of immunotherapy treatment. RNA was isolated from all samples by using RNA-STAT and DNase-treated with RQ1 RNase-free DNase as directed by the manufacturer. cDNA was generated using dT_{12-18} primers and murine Moloney leukemia virus reverse transcriptase. Semi-quantitative PCR amplification for β-actin and Mig was performed using the following primer pairs:

MuMig primers : forward (5′GATCAAACCTGCCTAGA3′);
reverse (5′CTTGAACGACGACGAC3′)

β-actin primers : forward(5′GTCCCTGTATGCCTCTG3′);
reverse(5′ATGAGGTAGTCTGTCAGGT3′)

Statistical analyses

To determine the statistical significance of the data, Tukey's Honestly Significant Difference test was performed at a P value set at 0.05. Tukey's test is a multi-comparison test that determines the statistical significance of three or more data sets and allows for unequal sample size (n) and sample variances [44].

Results

Treatment of mice with established B16melF10 lung metastases with transfected tumor cells plus IL-12 reduces metastatic disease

To test the combination therapy of IL-12, MHC class II, and CD80, we transfected the melF10 tumor with syngeneic MHC class II genes (Aa^b and Ab^b genes encoding the I-A^b class II molecule) plus the CD80 gene. Transfectants were screened by indirect immunofluorescence for class II and/or CD80 expression and cloned by limiting dilution.

Figure 1 shows the flow-cytometry profiles of melF10 cells transfected with MHC class II genes (melF10/A^b), the CD80 gene (melF10/B7), or both class II plus CD80 (melF10/A^b/B7) and stained with mAb for MHC class I, class II, or CD80. Wild-type melF10 and the transfectant cells have relatively low levels of endogenously encoded MHC class I molecules (H-2K^b and H-2D^b; 20-8-4 and 28-24-8 mAb; Fig. 1i–l and m–p respectively). I-A^b and CD80 are only expressed on those cell lines carrying these transgenes (Fig. 1b, d and g, h respectively).

Because our goal is to generate more effective therapeutic strategies, the therapeutic efficacy of the melF10 transfectants plus IL-12 was tested in mice with advanced established lung metastases. Syngeneic C57BL/6 mice were inoculated i.v. in the tail vein with 10^5 wild-type melF10 cells. Following i.v. injection the melF10 cells rapidly migrate to the lungs, and mice die from metastatic lung tumor within 3–4 weeks (V. Clements and S. Ostrand-Rosenberg, unpublished results). On day 7 after inoculation of wild-type tumor, immunotherapy was started. Each mouse was given one injection/week of 10^6 irradiated tumor cells or transfectants and three injections/week of 1 µg IL-12. Therapy was continued for 3 weeks; the mice were then sacrificed and the lungs removed, visually inspected, and weighed. Figure 2 shows the lungs of treated mice from one typical experiment and Table 1 shows the pooled results of three experiments in which tumor-bearing mice were treated with IL-12 ± melF10, melF10/A^b, melF10/B7, or melF10/A^b/B7 cells. By visual inspection of the lungs (Fig. 2) and by comparing the average of mean lung weights (Table 1), the greatest reduction in lung metastases can be seen in mice treated with melF10/A^b/B7 cells plus IL-12. Therapy with IL-12 alone also reduces lung metastases, as does therapy with melF10/A^b cells

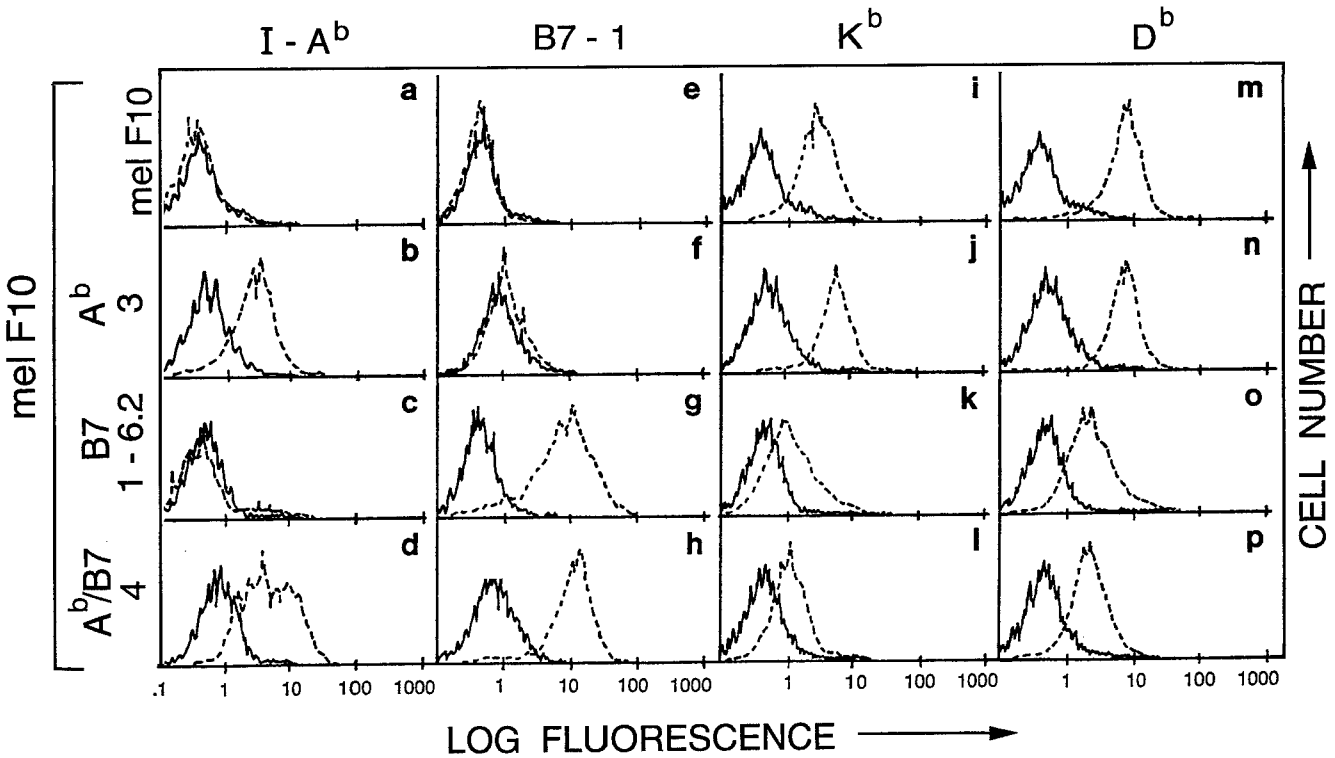

Fig. 1 Flow-cytometry profiles of melF10 and melF10 transfectants stained for MHC class I (H-2 K^b and H-2D^b), MHC class II (I-A^b), and B7.1 (CD80) molecules. *Dotted lines* staining by specific antibody plus fluorescent conjugate; *solid lines* staining by fluorescent conjugate alone

plus IL-12 or therapy with melF10/B7 cells plus IL-12. Therapy with transfectants alone does not have a measurable effect (Table 1). Statistical analysis of lung weights (Table 1), using Tukey's test at $P = 0.05$, demonstrates a significant difference between the treatment groups (IL-12 alone or IL-12 plus any cell combination) and the untreated group or the group treated with cells alone; however, the analysis does not demonstrate a statistically significant difference between the group receiving IL-12 alone and those treated with IL-12 plus any of the cell-based vaccines. The absence of statistical significance between these groups is most likely due to the approximation of metastatic cell content by

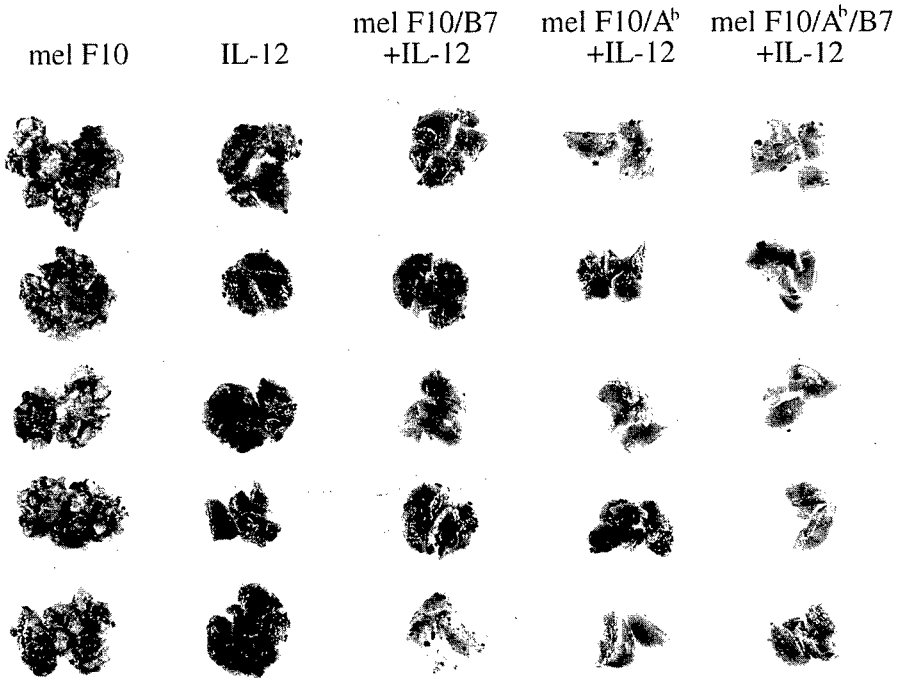

Fig. 2 Metastatic tumor cells in the lungs of C57BL/6 mice treated with cell and/or interleukin-12 (*IL-12*) therapies. Mice were inoculated i.v. on day 0 with wild-type melF10 tumor cells and therapy started on day 7. The therapy for each group is indicated. Following 3 weeks of therapy, lungs were excised. Each set of lungs is from an individual mouse

Table 1 Mice carrying established i.v. B16melF10 melanoma metastatses have reduced lung metastases following treatment with melF10/A^b/B7.1 plus interleukin-12 (*IL-12*). Syngeneic C57BL/6 J mice were challenged i.v. on day 1 with 10^5 wild-type B16melF10 tumor cells. Beginning 7 days after tumor inoculation, and continuing for the following 3 weeks, mice were inoculated i.p. once a week with therapeutic cells and three times per week i.p. with 1μg IL-12. Surviving mice were sacrificed on day 26 after initial tumor inoculation and lung weights determined

Treatment				Mean lung weight (mg) (±SD)
Therapeutic cells	Number mice per group	IL-12	T cell depletion	
–	9	–	–	1011 ± 213
melF10/A^b/B7.1	5	–	–	990 ± 169
–	10	IL-12	–	464 ± 124*
melF10	5	IL-12	–	313 ± 31*
melF10/A^b	5	IL-12	–	337 ± 55*
melF10/B7.1	5	IL-12	–	409 ± 35*
melF10/A^b/B7.1	15	IL-12	–	352 ± 205*
melF10/A^b/B7.1	5	IL-12	CD4 depleted	250 ± 41*
melF10/A^b/B7.1	5	IL-12	CD8 depleted	325 ± 13*

* These groups are statistically significantly different from the untreated groups and the groups treated with melF10/A^b/B7.1 cell vaccine alone (Tukey's test, $P = 0.05$)

lung weight, which can only detect gross differences. Therefore, by visual inspection, the therapy that shows the greatest reduction in lung metastases is treatment with IL-12 plus the MHC class II/CD80 vaccine.

Mice carrying established 4T1 mammary carcinoma metastases have reductions in metastatic disease following treatment with IL-12 plus 4T1 transfectants or wild-type tumor

To determine if the effect of therapy on MelF10 tumors is applicable to additional tumors, we performed similar experiments with the 4T1 tumor in which the number of metastatic cells can be very precisely quantified [32]. Syngeneic BALB/c female mice were inoculated in the mammary gland with 7×10^3 4T1 tumor cells and tumors allowed to develop for 3 weeks, at which time the primary tumors ranged from 1 mm to 8 mm in diameter. Previous experiments ascertained that, at this time and size of primary tumor, extensive metastatic disease is well established in the lungs, and that the number of metastatic cells in the lungs is proportional to the size of the primary tumor [32].

Therapy was started on day 21 after the initial tumor challenge and consisted of one injection per week of 10^6 irradiated tumor cells or transfectants (4T1, 4T1/A^d, 4T1/B7.1, or 4T1/A^d plus 4T1/B7.1) plus three injections/week of 1 μg IL-12/mouse. Mice were sacrificed after 3 weeks of therapy (day 42 after initial 4T1 inoculation) and lung tissue was dissociated into single-cell suspensions and plated in medium containing 6-thioguanine. Ten days later the number of clonogenic metastatic 4T1 cells was counted because 4T1 cells are resistant to 6-thioguanine and normal cells are killed by the drug [32].

Figure 3 shows the results of the 4T1 therapy experiments. Because we are interested in determining whether primary tumor size affects vaccine efficacy, the results are plotted as the number of metastatic cells in the lung at the end of treatment versus the size of primary tumor when the therapy is started. A line denoting a level of 10 000 tumor cells in the lungs is also included because almost all untreated mice contain more than 10 000 metastatic cells in their lungs after 42 days of primary tumor growth [32]. As shown in Fig. 3, therapy with unmodified tumor (Fig. 3A) or 4T1/A^d plus 4T1/B7.1 transfectants (Fig. 3B) minimally reduces the number of mice with more than 10 000 metastases in the lungs (69% and 67% respectively). Treatment with IL-12 alone (Fig. 3C) causes some reduction in the number of metastatic cells relative to control 4T1-treated mice (Fig. 3A); however, 41% of the IL-12-treated mice still have more than 10 000 metastatic cells in their lungs. In contrast, treatment with IL-12 plus any transfectant or IL-12 plus wild-type 4T1 results in only 11%–25% of mice having more than 10 000 metastatic cells in the lungs (Fig. 3D–G). To analyze the statistical significance of these results, we used the Tukey's test at $P = 0.05$. Because the individual treatment groups involving cells plus IL-12 (Fig. 3D–G) do not statistically differ from each other, the results of these groups were pooled. According to Tukey's test, the pooled results from the groups treated with cells plus IL-12 (Fig. 3D–G) are significantly different from those of groups receiving cell therapy alone (Fig. 3A, B), and IL-12 therapy alone does not give significantly different results from those of any treatment group. Maximum reduction of established, spontaneous 4T1 lung metastases, therefore, occurs following combination therapy of IL-12 plus irradiated tumor cells; however, wild-type tumor cells are as effective as transfectants in the combined therapy.

Combination IL-12 and cellular therapy affects the growth of smaller primary, solid tumors

Previous studies have demonstrated that IL-12 therapy mediates tumor regression of relatively small, primary,

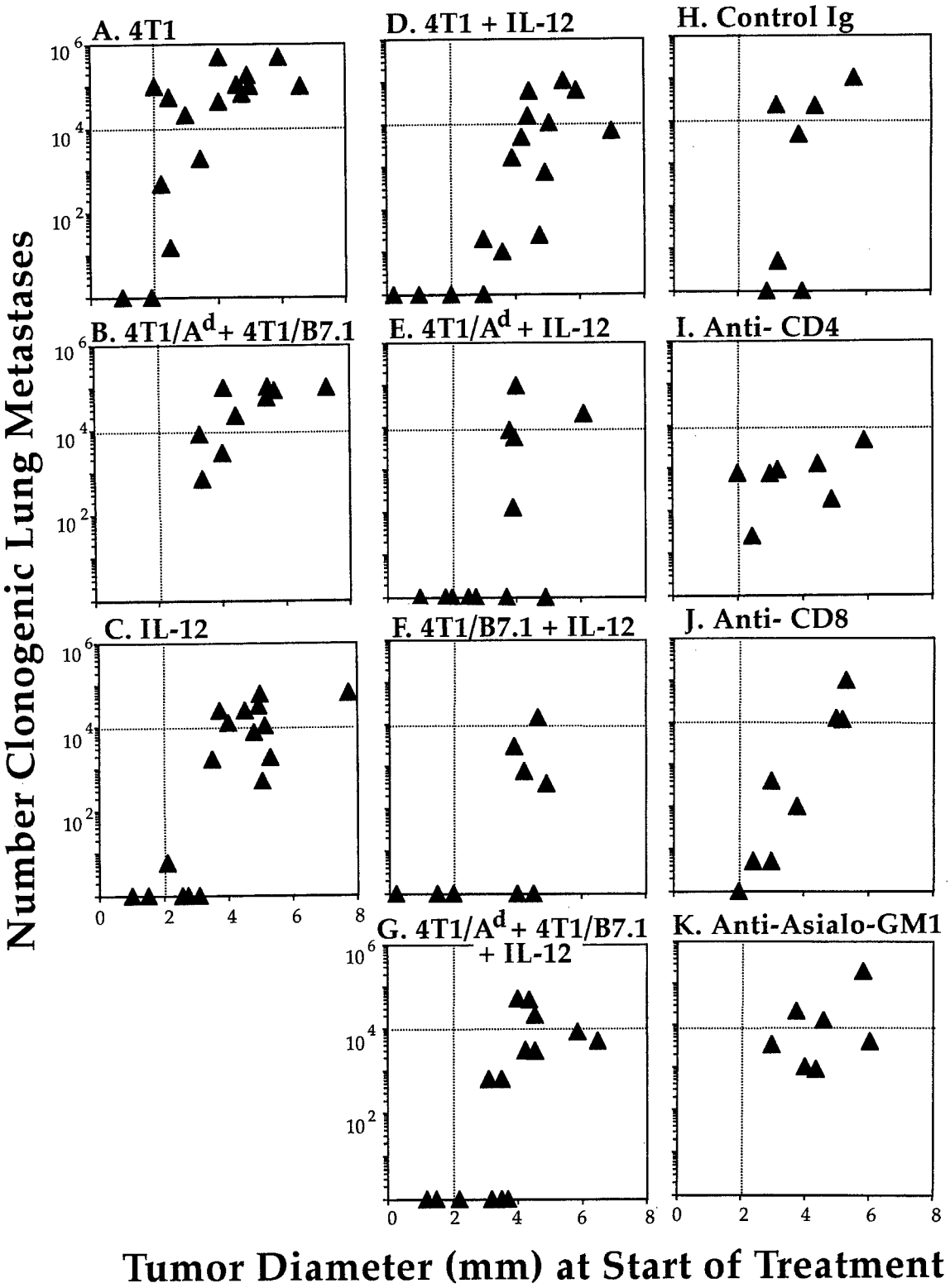

Fig. 3 Number of metastatic cells in BALB/c mice carrying established wild-type 4T1 metastases and treated with cell and/or IL-12 therapies. The therapy for each group is indicated within each panel. Mice were inoculated with wild-type tumor on day 0 and therapy started on day 21. Each symbol represents an individual mouse. To determine statistical significance, the number of clonogenic lung metastases were transformed to $\log_{10}$ values and analyzed using Tukey's test ($P = 0.05$)

solid tumors. To confirm these observations and to determine whether the presence of metastatic disease complicates the effect of IL-12 therapy on primary solid tumor, we have also analyzed primary tumor growth in the animals used for the results in Fig. 3. Primary tumor diameters were measured at the beginning of therapy and two or three times per week thereafter. At the end of

3 weeks of therapy, mice were categorized as follows: responding mice showed a marked reduction in primary tumor diameter, non-responding mice showed continued growth of primary tumor, partially responding mice showed no change in tumor size. To determine whether primary tumor burden at the start of therapy affects therapeutic outcome, mice were analyzed according to tumor diameter at the start of treatment. As shown in Table 2, mice treated with cells only (4T1 or 4T1/A^d + 4T1/B7.1) are uniformly non-responders. All mice with small tumors at the start of treatment (0–1.99 mm in diameter), regardless of the therapy (IL-12 alone or IL-12 plus cells), are responders. In contrast, mice with larger tumors (above 2 mm in diameter) at the start of therapy responded heterogeneously. For mice with tumors of 2–3.9 mm, IL-12 plus 4T1/A^d/B7.1 cells produced the largest number of responding mice; however all therapies, including IL-12 (IL-12 alone or IL-12 plus any cells), produced approximately equal responses if the results of responding and partially responding mice are pooled. In contrast, there were fewer responding and partially responding mice if therapy was started when tumors were 4–5.9 mm in diameter, and responses to IL-12 alone or IL-12 plus cells did not differ. Among mice starting with tumors more than 6 mm in diameter there were no responders or partial responders. In agreement with studies by other investigators, IL-12 therapy alone, therefore, mediates regression or partial regression of tumors less than 6 mm in diameter in a subset of treated mice. However, combination therapy of IL-12 plus tumor cells causes more complete responses for tumors that are 2–4 mm in diameter at the start of therapy.

Immunotherapy effect is not exclusively dependent on $CD4^+$, $CD8^+$, or NK cells

The concept of combining a cell-based immunization therapy with IL-12 was based on the hypothesis that IL-12 facilitates the development of T_{h1}-type $CD4^+$ T helper lymphocytes [18] that are activated by immunization with the MHC class $II^+B7.1^+$ tumor cells. These Th1 cells would, in turn, provide "help" to tumor-specific $CD8^+$ T lymphocytes and improved antitumor immunity would result. To test this hypothesis and to determine the involvement of T cells, mice undergoing immunotherapy were depleted of $CD4^+$ and $CD8^+$ T cells. Depletions were started prior to the first cycle of immunotherapy. These experiments were completed in both the melF10 and 4T1 systems, and the immunotherapy protocols for each tumor were identical to those used in the experiments of Table 1 (B16melF10 tumor) and Fig. 3 (4T1 tumor). As shown in Table 1, and Fig. 3H–J, depletion of $CD4^+$ or $CD8^+$T cells does not affect metastatic growth in mice treated with MHC class $II^+B7.1^+$ cells plus IL-12, indicating that neither of these populations by itself is responsible for the therapeutic effect.

In addition to its role in Th1 development, IL-12 also stimulates NK cell function [3]. Mice with the melF10 tumor and undergoing immunotherapy, therefore, were also tested for splenic NK cell levels and NK activity. Splenocytes of C57BL/6 mice carrying established melF10 metastases, and having received at least 2 weeks of immunotherapy were tested for NK1.1, $CD4^+$, $CD8^+$, $CD3^+$, and $B220^+$ expression. The percentages of $CD4^+$, $CD8^+$, and $B220^+$ splenocytes did not change following therapy with cells and/or IL-12 (data not shown). In contrast, as shown in Table 3, the percentage of $NK1.1^+$ splenocytes was statistically significantly higher in all treated mice than in naive untreated and tumor-bearing control mice (Tukey's $P = 0.05$). Therapy with any of the cell-based vaccines and/or IL-12, therefore, significantly increases the number of NK cells in the spleen.

Recent studies have attributed IL-12-mediated tumor rejection to a novel population of NK^+CD3^+ MHC-unrestricted effector cells [13]. Splenocytes from melF10-

Table 2 IL-12 alone and IL-12 plus cells mediate regression of primary tumors of 4 mm or less *ND* not determined; *R* responder, primary tumor diameter regressed during therapy; *PR* partial responder, primary tumor diameter remained the same during therapy; *NR* non-responder, primary tumor diameter continued to grow during therapy

Therapeutic cells	IL-12	No. responding mice/total mice treated, for mice having a tumor diameter at start of therapy (mm) of:			
		0–1.99	2–3.99	4–5.99	>6
4T1	–	2/2 NR	6/6 NR	7/7 NR	1/1 NR
4T1/A^d + 4T1/B7.1	–	ND	2/2 NR	6/6 NR	1/1 NR
–	IL-12	2/2 R	1/7 R 4/7 PR 2/7 NR	2/8 PR 6/8 NR	2/2 NR
4T1	IL-12	2/2 R	2/6 R 3/6 PR 1/6 NR	8/8 NR	1/1 NR
4T1/A^d	IL-12	1/1 (R)	3/8 R 4/8 PR 1/8 NR	2/2 NR	1/1 NR
4T1/B7.1	IL-12	2/2 (R)	1/3 R 1/3 PR 1/3 NR	2/5 R 3/5 NR	ND
4T1/A^d + 4T1/B7.1	IL-12	2/2 (R)	4/6 R 1/6 PR 1/6 NR	2/6 PR 4/6 NR	1/1 NR

bearing mice, therefore, were doubly stained for NK1.1 and CD3. As shown in Table 3, naive (non-tumor-bearing) and untreated tumor-bearing mice have lower levels of $NK1.1^+CD3^+$ cells than do mice receiving IL-12 and/or vaccine therapy. Statistical analysis using Tukey's test at $P = 0.05$ demonstrates statistically significant differences between the naive and untreated tumor-bearing groups, the groups treated with melF10 ± IL-12, and the group receiving melF10/A^b/B7 plus IL-12. Therapy of tumor-bearing mice with IL-12 and/or tumor cells, therefore increases NK^+CD3^+ cells, and the greatest increase is for mice treated with IL-12 plus the vaccine of class-II^+ $CD80^+$ cells, suggesting that NK^+CD3^+ cells may be involved in the therapeutic effect.

To determine if the increase in splenic NK levels resulted in an increase in functional NK cell activity, splenocytes from mice treated with melF10 and naive mice were tested in vitro as effector cells in NK assays using P815 cells as NK-resistant and YAC-1 cells as NK-sensitive targets. Tumor-free, untreated C57BL/6 mice were given poly(I,C) 24 h before assay to stimulate NK activity and served as positive controls for measuring NK functional activity. As shown in Table 3, treated mice did not have higher NK functional activity than untreated mice, while poly(I,C)-boosted mice showed NK activity: 30% killing at E:T ratios of 50:1 splenocytes:YAC-1 targets. Cytotoxicity against P815 cells was less than 5%. Mice treated with IL-12 and/or cell-based immunotherapies, therefore, have increased phenotypic levels of NK^+ splenocytes, but these splenocytes do not display increased cytotoxic function as measured in vitro.

To determine if NK cells are responsible for the antimetastatic effects of the cell-based vaccine plus IL-12 in the 4T1 system (Fig. 3D–G), BALB/c mice carrying 4T1 primary tumors were depleted of NK cells by anti-asialoGM1 polyclonal antibodies prior to initiation of 4T1/A^d plus 4T1/B7 plus IL-12 therapy. As shown in Fig. 3, treated mice depleted of NK cells (Fig. 3K) have more metastatic tumor cells in their lungs than non-NK-depleted mice (Fig. 3G, H); however, they do not have levels of metastatic cells as high as 4T1-treated mice (Fig. 3A). Statistical analysis of these data by Tukey's test does not demonstrate a significant difference between the non-depleted treated mice (Fig. 3G, H) and the asialoGM-1-depleted treated mice (Fig. 3K). NK cells, therefore, may mediate some of the therapeutic effect; however, other effector mechanisms are probably also involved.

Although neither T nor NK cells are exclusively responsible for the therapeutic effect, these cell populations may be functioning cooperatively to diminish growth of metastatic cells. To test this hypothesis, beige/nude/XID mutant mice, which are deficient in NK and T cells, were challenged with 4T1 tumor in the mammary gland and given therapy with 4T1 cells alone, IL-12 alone, or 4T1 cells plus IL-12 initiated on day 21. As shown in Fig. 4, therapy with IL-12 alone (Fig. 4B) or IL-12 plus 4T1 cells (Fig. 4C) resulted in 50% and 33% of mice having more than 10 000 metastatic cells in the lungs, respectively, whereas 100% of mice treated with 4T1 alone (Fig. 4A) had more than 10 000 metastatic cells in the lungs. When compared to immunocompetent mice treated with the same therapies (Fig. 3A, C, D respectively), however, the beige/nude/XID mice have a smaller reduction in the number of clonogenic lung metastases. Beige/nude/XID mice, therefore, respond to the therapy; however, the response is not as great as for immunocompetent mice, suggesting that NK and T cells are partially responsible for the therapy effect.

4T1 and MelF10 tumor cells are induced by IFNγ to express the chemokine Mig

Recent studies indicate that IL-12 and its downstream mediator IFNγ may regulate tumor growth by stimulating anti-angiogenic chemokines including monokine induced by IFNγ (Mig) and IFNγ-inducible protein 10 (IP-10) [12, 20, 40]. Because T and NK cells do not appear to be exclusively responsible for the anti-metastatic response, we hypothesized that chemokines

Table 3 Mice carrying established melF10 metastases and treated with IL-12 and/or cell vaccines have increased levels of splenic natural killer (NK) cells. The two group sizes are for NK1.1 and $NK1.1 + CD3^+$ groups respectively. Splenocytes from tumor-free, untreated mice given poly(I,C) 24 h prior to assay had NK cytotoxicity levels of 30% at 50:1 effector:YAC-1 ratios. The percentage cytotoxicity against P815 cells for all effectors was below 5%

Primary tumor	Number of mice in group	Therapy		NK cells in spleen (%)		Splenic NK activity (%) against YAC-1 at 50:1 E:T ratio
		Cells	IL-12	$NK1.1^+$	$NK1.1^+ + CD3^+$	
None	4, 4	–	–	3.8 ± 0.5	1.5 ± 0.2	ND
MelF10	4, 2	–	–	3.6 ± 1.5	2 ± 0.1	5
	4, 2	–	IL-12	14.9 ± 4.7*	7.4 ± 0.4**	2
	4, 4	MelF10	–	10.1 ± 0.4*	6.3 ± 0.7**	8
	4, 2	MelF10	IL-12	15.6 ± 9*	8 ± 2**	ND
	5, 2	MelF10/A^b/B7.1	IL-12	15.1 ± 8.8*	13 ± 0.3***	2

* Statistically significantly different from naive and tumor-bearing untreated control groups for NK1.1 analysis (Tukey's test $P = 0.05$)
** Statistically significantly different from naive and tumor-bearing untreated control groups and the group receiving melF10/A^b/B7.1 plus IL-12 therapy for $NK1.1^+ + CD3^+$ analysis (Tukey's test $P = 0.05$)
*** Statistically significantly different from all groups for $NK1.1^+ + CD3^+$ analysis (Tukey's test $P = 0.05$)

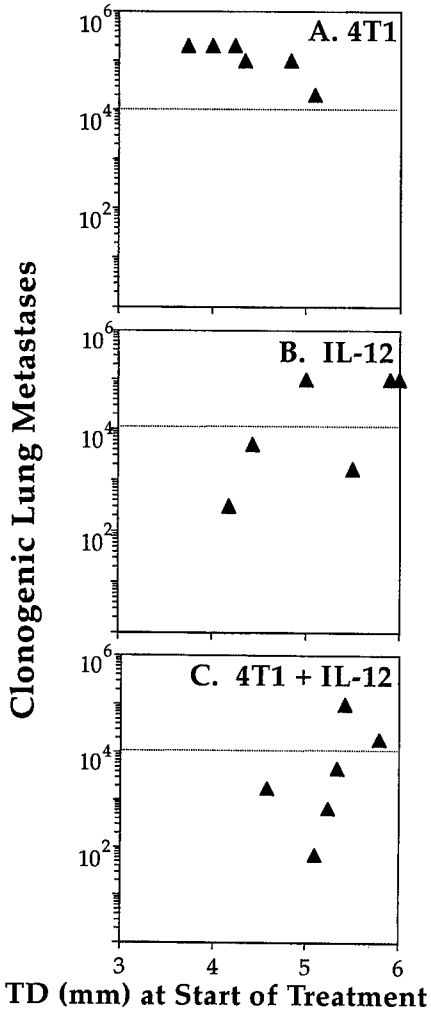

Fig. 4 Number of metastatic cells in CR:NIH-beige/nude/XID mice carrying established wild-type 4T1 metastases and treated with 4T1 cell and/or IL-12 therapy. Female CR:NIH-beige/nude/XID mice were inoculated with wild-type tumor on day 0 and therapy started on day 21. Each symbol represents an individual mouse

such as Mig and IP-10 might be involved. To determine if our combination therapy stimulates Mig and/or IP-10 expression, RNA was prepared from the lungs of tumor-bearing, treated mice, reverse-transcribed, and PCR-amplified using Mig-specific and IP-10-specific PCR primers. To semi-quantify the amount of chemokine, PCR was performed for 26, 28, or 30 cycles. Although IP-10 was not expressed in the lungs (data not shown), Mig mRNA is detectable by PCR in the lungs of 4T1 tumor-bearing mice 4 h, 7 days and 21 days after initiation of therapy (Fig. 5C). Reverse transcription (RT)/PCR using β-actin primers confirmed the integrity of the RNA from treated mice (Fig. 5B). In contrast, lungs from untreated, tumor-free naive mice only showed a very faint band for Mig after 30 cycles, demonstrating that they have much lower levels of Mig in their lungs (Fig. 5A). Since naive lung does not express high levels of Mig, it is possible that the metastatic 4T1 cells are responsible for producing the Mig.

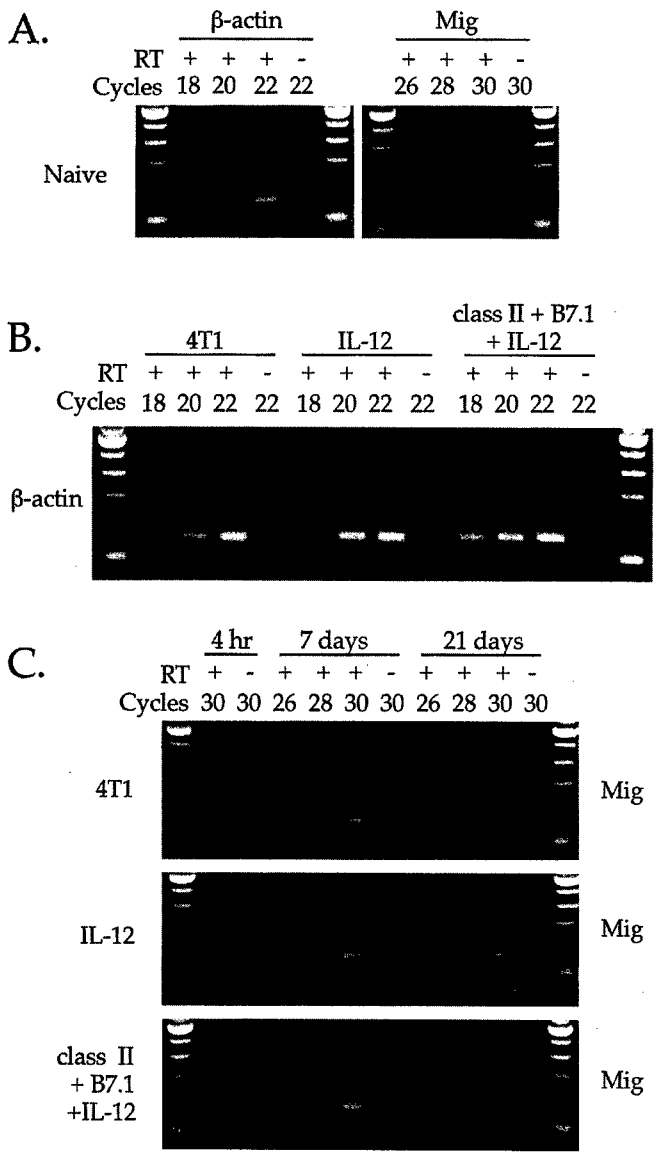

Fig. 5A–C mRNA for the monokine induced by interferon γ (Mig) is expressed in the lungs of BALB/c mice carrying 4T1 tumors. BALB/c mice were inoculated in the mammary gland with 7×10^3 4T1 cells on day 0 and therapy started on day 21. Lungs were removed 4 h, 7 days, and 21 days after therapy was started and lung RNA was isolated, reverse-transcribed, and amplified by the polymerase chain reaction using Mig-specific or β-actin-specific primers for the indicated number of cycles

To determine if the tumor cells synthesize Mig and therefore produce the Mig detected in the lungs, 4T1 and MelF10 cells were cultured with and without IFNγ for 2 h in vitro and Mig and β-actin expression analyzed by reverse PCR. As shown in Fig. 6, IFNγ induces expression of Mig in both 4T1 and MelF10 tumor cells. The resident tumor cells, therefore, may be generating the anti-angiogenic/chemoattractant factor.

Discussion

Several studies have reported a synergistic therapy effect when CD80-transfected tumor cells are combined with

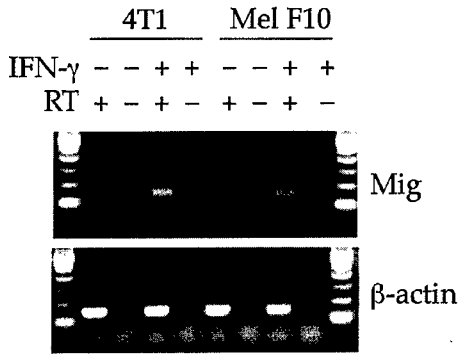

Fig. 6 4T1 and B16melF10 tumors are induced in vitro by interferon γ (IFNγ) to express Mig. RNA from 4T1 and melF10 tumor cells cocultured with or without IFNγ for 2 h was reverse-transcribed and amplified using Mig-specific or β-actin-specific primers

IL-12 and used as a vaccine to protect mice against a subsequent or simultaneous challenge of tumor [8, 9, 31, 45]. Other studies have shown therapeutic efficacy of CD80-transfected tumor cells combined with IL-12 in the treatment of very recently established experimental lung metastases [11, 34], or longer-established primary, solid tumors [33]. With the exception of one study, which reported that IL-12 up-regulated B7 expression on host antigen-presenting cells [14], all of these studies demonstrated more effective immunity when IL-12 and CD80 were combined.

Although all of these studies showed potent antitumor activity and reductions in tumor growth, only one of the reports examined spontaneous metastatic disease [22], and none of the reported studies addressed longer-term experimental or spontaneous metastatic disease. In addition, the previously reported studies have focused exclusively on activation of $CD8^+$ T cells, although CD80 and IL-12 are potent activators of $CD4^+$ T cells [23, 25]. Since much metastatic disease is poorly responsive to conventional treatments and immunotherapy is a potential alternative treatment, we have incorporated the combined therapy of CD80 plus IL-2 with our previously developed approach targeting the activation of $CD4^+$ T cells, and tested the combined approach in two mouse tumor systems in which metastatic lesions have been established longer and/or arise spontaneously.

In both the B16melF10 and 4T1 tumor systems the combination IL-12 plus cell-based vaccine therapy reduces metastases. The role of transfected tumor cells, however, may differ between the two tumors. For the B16melF10 tumor, therapy with IL-12 plus MHC class II, CD80-transfected tumor cells may provide greater protection than therapy with IL-12 plus non-transfected tumor cells. Although the increased protection is clearly apparent from visual inspection of the lungs of treated mice (Fig. 2), analysis of lung weights, using the appropriate statistical formula, does not demonstrate a significant difference between the groups treated with IL-12 alone and those receiving IL-12 plus cells. Lung weight is only an approximation of metastatic content, however, and it is unlikely that small, but significant, differences in numbers of metastatic cells will be detected by this measurement. Lung weights, therefore, probably do not accurately measure metastatic cells. Although enumerating the number of metastatic nodules in the lungs could also quantify metastatic spread, this measurement is also unlikely to reflect small, but significant differences, in the number of metastatic cells since individual nodules are of different size and contain different numbers of cells. The differences seen in Fig. 2, between the group receiving IL-12 alone and those treated with IL-12 plus vaccines, therefore, are likely to represent genuine differences in therapeutic efficacy of the different combinations of therapeutic agents even though the statistical analysis of lung weights does not indicate that these differences are significant. For the 4T1 tumor, treatment of established metastases with IL-12 plus any transfectant or wild-type tumor is equally efficacious, and the combination therapy of IL-12 plus cells is significantly more effective than therapy with IL-12 or cells alone.

Although numerous other studies have assessed effects of IL-12 on primary or i.v. induced experimental metastases, few animal models are suitable for analyzing the effects of IL-12 on individual animals with both primary tumor and metastatic disease. As shown in Table 4 for the 4T1 tumor, although therapy with IL-12

Table 4 Summary of primary and metastatic 4T1 tumor growth in BALB/c mice treated with IL-12 plus cell-based vaccine. In the case of the primary tumor, mice are classified as responders, partial responders, or non-responders. The diameter of the primary tumor decreased in responding mice, remained unchanged in partially responding mice, and increased in non-responding mice. In the case of metastases (*Mets*), the mice are classified as either responders or non-responders. Responding mice have 10 000 or fewer clonogenic metastatic tumor cells in their lungs; non-responding mice have more than 10 000 metastatic cells. Results for the *Cells + IL-2* group are pooled from groups treated with IL-12 plus $4T1/A^d$, 4T1/B7.1, or 4T1 tumor cells

Therapy	Responding plus partially responding mice (%) having a primary tumor diameter at start of therapy (mm) of:							
	0–1.99		2–3.99		4–5.99		≥6	
	Primary tumor	Mets	Primary tumor	Mets	Primary tumor	Mets	Primary tumor	Mets
4T1 cells	0	100	0	50	0	0	0	0
IL-12	100	100	71	83	25	43	0	0
Cells + IL-12	100	100	82	100	19	52	0	66

plus cells does not affect growth of primary tumors of more than 4 mm, this therapy still reduces growth of metastatic cells in mice with primary tumors of this size. The combined immunotherapy of cells plus IL-12, therefore, is effective against metastases even when the host animal has a primary tumor that does not respond to the therapy.

Since IL-12 stimulates Th-1 lymphocyte activity [18, 23, 25], our expectation was that both $CD4^+$ and $CD8^+$ T cells would be required for the immunotherapy effect. Contrary to this expectation, neither T cell population alone was uniquely responsible for the therapy effect. Because IL-12 also stimulates NK cell activity, it was also anticipated that increased NK activity may contribute to the therapeutic effect. Although splenocytes with an NK phenotype, as measured by immunofluorescence, were increased in treated mice, there was no concomitant increase in NK functional activity, as measured by in vitro NK assay in the melF10 system. However, mice depleted in vivo for NK cells in the 4T1 system have slightly higher levels of metastatic tumor, suggesting that NK cells are involved in the therapeutic effect. Further confounding the identification of the effector cells in the combination therapy is the observation that mice deficient in NK and T cells (CR:NIH-beige/nude/XID mice) remain at least partially responsive to the therapy in the 4T1 system, suggesting that effector cells/mechanisms other than T and NK cells (e.g. neutrophils, eosinophils, macrophages, anti-angiogenesis factors, etc.) may also be involved in the therapeutic effect.

As recently suggested by several other studies, IL-12 stimulates IFNγ production, which, in turn, stimulates expression of chemokines such as Mig that may either directly or indirectly affect tumor growth [12, 20, 40]. Mig and/or IL-12 could be affecting tumor growth by at least three mechanisms. (1) Because Mig is a chemoattractant for T cells and NK cells [15] it may facilitate migration of these effectors to the lungs where they mediate tumor cell destruction. (2) Because of their anti-angiogenic activity, Mig and IL-12 may directly or indirectly limit tumor-mediated angiogenesis [5, 12, 15, 20, 36, 43]. (3) Because IL-12 causes tumor necrosis, the lung metastases may become necrotic [37]. Collectively, the antibody depletion, chemokine induction, and experiments with immunodeficient mice in this paper suggest that the combined IL-12 and cellular vaccine therapy induces a combination of effector cells and effector molecules, including T cells, NK cells, neutrophils, and chemokines that synergistically diminish growth of lung metastases.

Mice treated in these studies had extensive tumor burdens and metastatic disease, much more advanced than mice commonly used in experimental immunotherapy protocols. Although the precise mechanism and contributions of each effector cell type and/or factor responsible for the anti-therapeutic effect are unclear, the combined use of tumor cells/transfectants plus IL-12 produces a more potent antitumor effect than either IL-12 or tumor cells/transfectants alone and the combined use of these reagents in clinical immunotherapy protocols should be considered.

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ORIGINAL ARTICLE

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Tumor-derived Fas ligand induces toxicity in lymphoid organs and plays an important role in successful chemotherapy

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Abstract Recent studies have suggested that Fas ligand ($FasL^+$) tumor cells can induce apoptosis in Fas^+ T cells. However, the effect of growth of $FasL^+$ tumors in vivo, on lymphoid tissues of the host is not clear and therefore was the subject of this investigation. Injection of $FasL^+$ LSA tumor caused a significant decrease in cellularity of the thymus and spleen, resulting from marked apoptosis, in syngeneic C57BL/6+/+ (wild-type) but not C57BL/6-*lpr*/*lpr* (Fas-deficient) mice. The tumor-induced toxicity resulted from tumor-derived rather than host-derived FasL, inasmuch as LSA tumor growth in C57BL/6-*gld*/*gld* (FasL-defective) mice, induced marked apoptosis and toxicity in the thymus and spleen. The LSA tumor growth induced a significant decrease in the percentage of $CD4^+CD8^+$ T cells in the thymus of C57BL/6+/+ mice and an increase in the percentage of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T cells. Of the four subpopulations tested, the $CD4^+CD8^+$ T cells showed maximum apoptosis. The LSA ($FasL^+$) but not P815($FasL^-$) tumor cell lysates and culture supernatants induced marked apoptosis in Fas^+ thymocytes, when tested both in vitro and in vivo. The LSA-tumor-induced apoptosis in vitro was inhibited by antibodies against FasL or by caspase and other inhibitors of apoptosis. Chemotherapy of LSA-tumor-bearing C57BL/6+/+ mice at advanced stages of tumor growth failed to cure the mice, whereas, more than 80% of LSA-tumor-bearing C57BL/6-*lpr*/*lpr* mice, similarly treated, survived. Together, the current study demonstrates that FasL produced by LSA tumor cells is functional in vivo and can cause severe toxicity in lymphoid organs of the host. Also, Fas/FasL interactions may play an important role in the successful chemotherapy of FasL-bearing tumor.

Key words Apoptosis · Fas ligand · Chemotherapy · Immunotoxicity

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Introduction

There are a number of mechanisms by which tumor cells may evade the actions of the immune system of the host. Recently the role played by Fas ligand (FasL) expressed on tumor cells in killing the Fas^+ antitumor immune effector cells, has attracted considerable attention [19]. Several tumors including, colon carcinomas [8], hepatocellular carcinoma [14] and astrocytoma [11] have been shown to express FasL. The FasL expressed on tumor cells is functional inasmuch as such tumor cells have been shown to deliver a death signal to Fas^+ target cells in vitro [2]. Recent studies from our laboratory demonstrated that a $FasL^+$ tumor cell line induced apoptosis in a Fas^+ tumor-specific cytotoxic T lymphocyte (CTL) line [20]. These data suggested that FasL-based killing of CTL by the specific tumor cells may lead to immune evasion by the tumor cells. Furthermore, the killing of Fas^+ CTL by the $FasL^+$ tumors may constitute a major limiting factor in successful immunotherapy of FasL-bearing cancer.

Despite such indirect evidence, it is not clear whether the growth of a Fas^+ tumor, in vivo would induce apoptosis in Fas^+ cells of the host. FasL is known to be expressed in both membrane-bound and soluble form [15]. However, the soluble form of FasL is less cytotoxic than the membrane-bound form [17]. In the current study, the effect of tumor growth on lymphoid organs was investigated in the murine host. The data demonstrated that tumor growth caused marked atrophy in the thymus and spleen of Fas^+ but not Fas-deficient mice, resulting from induction of apoptosis. Also, the Fas-FasL-based interactions between the host and the tumor cells played a critical role in determining the success of chemotherapy.

Materials and methods

Mice

Female C57BL/6+/+ (wild-type), C57BL/6-*lpr*/*lpr*, C57BL/6-*gld*/*gld* and DBA/2 mice, 4 weeks old, were purchased from Jackson Lab (Bar Harbor, Me.).

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Cooperativity of *Staphylococcal aureus* Enterotoxin B Superantigen, Major Histocompatibility Complex Class II, and CD80 for Immunotherapy of Advanced Spontaneous Metastases in a Clinically Relevant Postoperative Mouse Breast Cancer Model[1]

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ABSTRACT

One of the leading causes of death for women is metastatic breast cancer. Because most animal tumors do not accurately model clinical metastatic disease, the development of effective therapies has progressed slowly. In this study, we establish the poorly immunogenic mouse 4T1 mammary carcinoma as a postsurgical animal model. 4T1 growth characteristics parallel highly invasive human metastatic mammary carcinoma and, at the time of surgery, the extent of disease is comparable with human stage IV breast cancer. Progress in understanding the immune response has led to innovative immune-based anticancer therapies. Here, we test in this postsurgical model, a novel cell-based vaccine, combining MHC class II, CD80 (B7.1), and SEB superantigen. Effective treatment of tumor-bearing mice with this immunotherapy requires expression of all three molecules. Mean survival time is extended from 5–7.5 weeks for control-treated mice to 6–10.5 weeks for therapy-treated mice. Increased survival is accompanied by a maximum of 100-fold decrease in clonogenic lung metastases. These therapeutic effects are particularly noteworthy because: (*a*) the postoperative model demonstrates that early metastases responsible for morbidity are established by 2 weeks after tumor inoculation with 7×10^3 parental 4T1 cells into the mammary gland; (*b*) the immunotherapy is started 4 weeks after tumor inoculation when the mice contain extensive, pre-established, disseminated metastases; and (*c*) $CD4^+$ and $CD8^+$ T cells are required for the effect.

INTRODUCTION

As a result of recent discoveries and advances in immunology and molecular cloning, many novel immunotherapeutic strategies for the treatment of cancer are being developed (1, 2). Most of these strategies are focused on eliminating primary tumors, which are frequently successfully treated by conventional methods, such as surgery. In contrast, few immunotherapeutic approaches are targeting disseminated metastatic disease, for which conventional therapies frequently have limited success. Development of therapies for the treatment of metastatic disease is complicated by the shortage of animal models for spontaneously metastatic cancers. We recently described a novel cell-based vaccine for the therapy of metastatic disease and tested it using the mouse 4T1 mammary carcinoma model. The 4T1 tumor shares many characteristics with its human counterpart (3), making it an excellent animal model.

In most clinical situations, primary mammary tumors are cured by surgery, yet approximately 33% of women successfully treated for primary tumors die subsequently from spontaneous metastatic disease (4). To further refine the 4T1 system and to more closely parallel clinical disease, we have now developed a postsurgical model of the 4T1 mammary tumor. In this model, mice receive inoculations s.c. in the abdominal mammary gland and the primary tumor is allowed to grow progressively, become extensively vascularized, and metastasize. The primary tumor is then surgically resected, and therapy with the cell-based vaccines is initiated.

The cell-based vaccines consist of tumor cells transfected with syngeneic MHC class II ($I\text{-}A^d$) and CD80 (B7.1) costimulatory molecule genes and were designed to enhance activation of tumor-specific $CD4^+$ T lymphocytes via improved presentation of tumor-encoded class II-restricted epitopes. Although $CD8^+$ T lymphocytes have been traditionally the focus of immunotherapy approaches, accumulating results have demonstrated that $CD4^+$ T lymphocytes also play a critical role in effective antitumor immunity (5–9). Whereas our previous vaccines showed significant reduction of established, spontaneous metastatic tumor, the antitumor response was limited to small burdens of metastatic cells and did not completely eliminate metastases (3). In addition, we did not assess the effects of immunotherapy on survival. Furthermore, the vaccine was tested in mice with metastatic disease and carrying intact primary tumor so the model did not mimic the clinical situation in which primary tumor would have been surgically removed before initiation of immunotherapy. We now report a second-generation cell-based vaccine that is significantly more effective than the original vaccine for the treatment of spontaneous 4T1 metastatic mammary cancer and that is tested in a postsurgical model. The new vaccine incorporates a gene encoding the bacterial toxin SEB[3]. SEB is a sAg that when complexed with MHC class II molecules on APCs is a potent polyclonal activator of $CD4^+$ T lymphocytes (10, 11). Although $CD4^+$ T-cell activation by SEB is not antigen specific, we reasoned that the addition of SEB to the MHC class II/CD80 vaccine will provide additional activation signals to the $CD4^+$ T cells that have been activated in an antigen-specific fashion by the MHC class II^+ $CD80^+$ vaccinating cells.

MATERIALS AND METHODS

cDNA Expression Vectors. The expression vectors pHβ-Apr-1-neo containing MHC class II ($I\text{-}A\alpha^d$, $I\text{-}A\beta^d$) and mouse B7.1 have been described previously (3). The *SEB* gene (12) was subcloned into the *SalI*/*Bam*HI site of the pHβ-Apr-1-neo expression vector. The final construct, pHβ-SEB-neo, contains the amino acid sequence for the mature SEB protein minus the signal peptide and confers resistance to G-418. The pZeoSV2 plasmid was purchased from Invitrogen (San Diego, CA).

Animals, Cell Lines, and Transfectants. Female BALB/c and BALB/c *nu/nu* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and/or bred in the University of Maryland Baltimore County animal facility and used at 8 weeks of age. 4T1, a 6-thioguanine-resistant cell line derived from a BALB/c spontaneous mammary carcinoma (13), was kindly supplied

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[3] The abbreviations used are: SEB, *Staphylococcal aureus* enterotoxin B; sAg, superantigen; APC, antigen-presenting cell; TD, mean tumor diameter.

by Dr. Fred R. Miller (Michigan Cancer Foundation, Detroit, MI) and grown in culture as described previously (3). Transfectants were made to express MHC class II and CD80, or SEB by using lipofectin (Life Technologies, Inc.) according to manufacturer's instructions. Cells were selected with 400 μg/ml G-418 (Life Technologies, Inc.) or 200 μg/ml zeocin (Invitrogen), cloned by limiting dilution, stained for surface antigen expression, and analyzed by flow cytometry, as described previously (3).

SEB Assay. Naive BALB/c spleen cells ($5–10 \times 10^5$) were cultured in serial dilutions of transfectants' supernatants or purified SEB (Sigma Chemical Co., St. Louis, MO), as indicated. To demonstrate specific SEB activity, a polyclonal rabbit antibody against SEB (Sigma Chemical Co.) was added to cultures, as indicated. After 3 days in culture, spleen cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent, as described previously (14).

Surgery. All surgical supplies and surgical equipment were purchased from Henry Schein Veterinary & Medical Supply Catalogue (Melville, NY) and Roboz (Rockville, MD), respectively, unless indicated otherwise. Before surgery, animals were weighed and anesthetized with i.p. injections (0.02 ml/g body weight) of 2.5% recrystalized avertin (2,2,2-tribromomethanol; Sigma Chemical Co.). Once the animals were unconscious, the tumor-bearing abdominal area was prepared and sterilized for surgery by shaving with Oster Finisher Trimmer, followed by 2 washes each with diluted Nolvasan surgical scrub (chlorhexidine) and isopropanol. Tumors were resected with sterilized surgical instruments, removing the smallest amount of skin tissue as possible. Wounds were closed with either Nexaband liquid or stainless steel 9-mm wound clamps with a Mikon autoclamp applier, as necessary. Wound clamps were removed 10 days after surgery with a Mikon autoclamp remover. Mice were monitored for survival, and those that died from surgery (within 1–4 days after surgery, survival rate of 67–80%) were not included in the experiment. All mice were autopsied at the time of death to confirm the presence of lung metastases as well as recurrence of the primary tumor.

Tumor Challenges, Metastases Assays, and *in Vivo* Depletions. Mice were challenged s.c. in the abdominal mammary gland with (7×10^3/50 μl) parental 4T1 tumor cells. Primary tumor growth and spontaneous metastases were measured as described previously (3). Depletions of $CD4^+$ and $CD8^+$ T cells were performed as described previously (15). Splenocytes of all depleted mice were checked by immunofluorescence for depletion at the conclusion of the experiment. Mice depleted for $CD4^+$ or $CD8^+$ T cells had <4% or 7% of $CD4^+$ T cells or $CD8^+$ T cells, respectively.

Statistical Analyses. To determine the statistical significance of the data, the Tukey's Honestly Significant Difference Test was performed at a P set at 0.05. The Tukey's test is a multicomparison test that determines the statistical significance of data sets of size 3 or greater and allows for unequal sample size (n) and sample variances (16). To determine the statistical significance of the effects of immunotherapy on primary tumor growth, the Student's t test for unequal variances (Microsoft Excel, version 5.0) was performed.

RESULTS

Tumor Lethality Is Due to Early Metastases. Our previous studies have demonstrated that the BALB/c-derived 4T1 mammary carcinoma is a poorly immunogenic and highly malignant tumor that rapidly and spontaneously metastasizes throughout the body in a pattern similar to human breast cancer (3). For example, primary 4T1 tumors that have been established for 2–3 weeks in BALB/c mice typically metastasize to the lymph nodes, lungs, and livers in 86%, 79%, and 20% of mice, respectively, and the numbers of micrometastatic cells found in these organs range between 2–57, 1–338, and 0–1, respectively. In addition, as the primary tumors age (*i.e.*, by 4–5 weeks), the incidence of metastases in the lungs, livers, and now brains increases to 91%, 82%, and 36% of mice, respectively, and the range of metastatic cells for these organs is between 6–250,000, 7–7800, and 1–116, respectively (3).

As shown in Fig. 1, 4T1 is also similar to human mammary carcinoma in that morbidity is due to outgrowth of spontaneous micrometastatic tumor cells that migrate to distant organs relatively early (week 2) during primary tumor growth. Groups of female

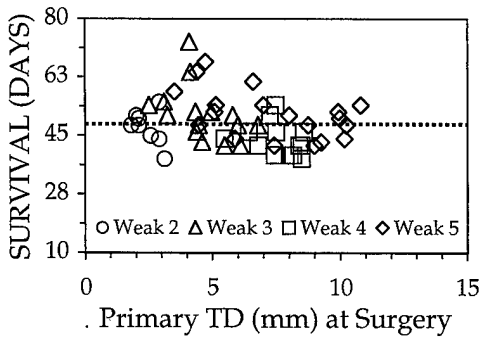

Fig. 1. Early spontaneous metastases are responsible for mortality. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 live wild-type 4T1 cells. Primary TDs were measured, and tumors were surgically resected at varying times after inoculation (weeks 2–5). Each point represents the survival time in days after primary tumor challenge for an individual mouse.

BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 wild-type 4T1 tumor cells. Starting at 2 weeks after challenge and continuing at 1-week intervals, TDs were measured, primary tumors were surgically removed, and mice were followed for survival times. Because we were interested in knowing whether primary tumor size affected survival time, the data have been plotted as tumor size (mm) at the time of surgery *versus* the number of days the mice survived after 4T1 tumor challenge. As shown in Fig. 1, the average survival time of 55 of 58 mice was 48.9 (±7.4) days, whereas the remaining 3 mice, whose tumors were <3 mm in diameter at the time of surgery, lived >90 days and did not die of metastatic cancer. Surprisingly, all mice that died from spontaneous metastatic disease showed approximately the same mean survival time regardless of the size of the primary tumor at the time of surgery. These results demonstrate that lethal metastasis is established as early as 2 weeks after inoculation of primary tumor, that the mean survival time is 7 weeks, and that surgical removal of primary tumor does not change these kinetics.

The surgical experiments of Fig. 1, combined with our previous studies (3), demonstrate that the 4T1 system is comparable with human stage IV breast cancer. Human breast cancer at stage IV is characterized by several diagnostic factors: (*a*) the presence of edema and ulcerations of the skin in and around the tumor burden; (*b*) extension of the primary tumor to the chest cavity lining; (*c*) presence of metastatic cells in the draining lymph nodes; and (*d*) presence of metastases in distant organs (4). This postsurgical 4T1 system exhibits all of these characteristics. Metastases are present in the draining lymph nodes and distant organs as early as week 2 and progress into more advanced metastatic disease with time (3). All of the resected 4T1 tumors, regardless of their size at the time of surgery, were highly vascularized. The primary tumors displayed edema when there was a TD ≥4 mm and ulcerations of the skin in approximately 70% of tumors regardless of size (data not shown). Most tumors extended to the lining of the peritoneal cavity, whereas invasion through the peritoneal lining was less frequent (<5%) and only occurred when primary TD was large (5–6 mm; data not shown). Therefore, at the time of surgery, the mouse 4T1 tumors are comparable with stage IV human breast cancer and are a much more rigorous animal model for the development of effective therapies than other experimental systems reported in the literature.

Postoperative Treatment of Mice with Transfectants Expressing MHC Class II, CD80, and SEB Increases Survival. Previously, we have shown that therapy with transfectants expressing MHC class II or CD80 reduced metastatic disease in a model where the primary 4T1 tumor remained *in situ* and had been established for 9–14 days.

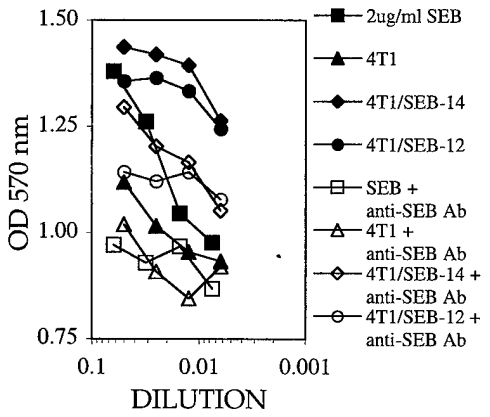

Fig. 2. Supernatants from 4T1/SEB transfectants stimulate proliferation of spleen cells. Naïve BALB/c splenocytes were cocultured with soluble SEB starting at a concentration of 2 μg/ml (■,□) or supernatants from parental 4T1 (▲,△), 4T1/SEB-12 (●,○), or 4T1/SEB-14 (◆,◇) transfectants in the presence (*open symbols*) or absence (*filled symbols*) of antibody specific for SEB.

The success of this treatment, however, was limited to small tumor burdens and did not completely eliminate spontaneous metastases (3). A potential problem with this earlier therapy is that the transfectants did not coexpress MHC class II and CD80, and previous data using a mouse sarcoma showed that coexpression of these molecules is synergistic (15). We have, therefore, generated 4T1 transfectants that coexpress MHC class II and CD80 as detected by indirect immunofluorescence staining (data not shown) to test this hypothesis. To further increase the potency of the vaccine, we have combined the MHC class $II^+/CD80^+$ double transfectants ($4T1/A^d/B7.1$) with SEB^+ transfectants (4T1/SEB), reasoning that SEB may provide additional proliferation signals to the tumor-specific T cells activated via the MHC class II/CD80 interaction.

SEB expression was tested by coculturing supernatants of transfectants with naive BALB/c spleen cells and monitoring lymphocyte proliferation. To determine a relative amount of SEB secretion, splenocytes were also cultured with soluble SEB. As shown in Fig. 2, supernatants from two independent clones (4T1/SEB-12 and 4T1/SEB-14) stimulated splenocyte proliferation as efficiently as soluble SEB at a concentration of 2 μg/ml. This activity was reduced on the addition of a polyclonal anti-SEB antibody, demonstrating that the spleen cell proliferation was due to SEB expression by the 4T1 transfectants. Supernatants from parental 4T1 cells as well as supernatants from 4T1 cells transfected with empty vector (4T1/neo) did not induce proliferative responses (Fig. 2 and data not shown). Therefore, the 4T1/SEB transfectants secrete SEB, which induces splenocyte proliferation comparable with proliferation induced by soluble exogenously added SEB.

Vaccines such as the 4T1 transfectants are likely to be most useful for the treatment of disseminated spontaneous metastatic disease because primary tumors usually can be eliminated by surgery. Therefore, we have tested the combination vaccine in mice with established, disseminated spontaneous metastases following surgical removal of the primary tumor. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 wild-type 4T1 tumor cells. The tumors were allowed to grow and metastasize for 3 weeks, at which time the primary tumor burden was measured and surgically resected. The experiments in Fig. 1 and our previously published results (3) established that at this stage and size of primary tumor the mice have very extensive disseminated metastases. At the time of surgery (3 weeks after primary tumor challenge), the primary TD in each treatment group ranged between 3.5 mm and 5.7 mm. The extent of spontaneous metastatic cancer at this time is significantly more advanced than that tested in other immunotherapy experiments. Therapeutic injections of irradiated vaccine cells (1×10^6 total) were started 1 week after surgery (*i.e.,* 4 weeks after initial tumor challenge) and were administered every 3–5 days for the duration of the experiment. Because we are interested in determining whether primary tumor size affects vaccine efficacy, the results in Fig. 3 are plotted as the survival time *versus* the size of the primary tumor at the time of surgery. A *line* denoting the average survival time of the 4T1-treated control group (45 days) is included to demonstrate the effects of the vaccine on survival. The survival time in days for 4T1-treated (Fig. 3*A*), 4T1/SEB-treated (Fig. 3*B*), $4T1/A^d/B7.1$-treated (Fig. 3*C*), and $4T1/SEB+4T1/A^d/B7.1$-treated (Fig. 3*D*) animals was 35–52, 40–59, 47–54, and 41–74 days, respectively. Statistical analyses using the Tukey's Honestly Significant Difference Test revealed that only the treatment with a 1:1 mixture of $4T1/A^d/B7.1+4T1/SEB$ cells significantly increases the survival time of mice with established wild-type metastatic disease ($P = 0.05$). Treatment with either 4T1/SEB alone or $4T1/A^d/B7.1$ alone does not significantly increase survival. Therefore, therapy with this cell-based vaccine requires expression of all three molecules to extend mean survival time from 5–7.5 weeks for control-treated mice to 6–10.5 weeks for therapy-treated mice. Although this increase in survival time is relatively small, it is statistically significant and compelling because the immunotherapy was started at week 4 and untreated and/or 4T1-treated mice begin to die as early as 5 weeks after tumor challenge.

Increase in Survival Correlates with Reduction of Metastatic Cancer. To demonstrate that the increase in survival was due to a reduction of spontaneous metastatic cancer, lungs from therapy-treated animals were harvested and the number of clonogenic metastases was quantitated as described previously (3). Female BALB/c mice were challenged s.c. in the abdominal mammary gland with

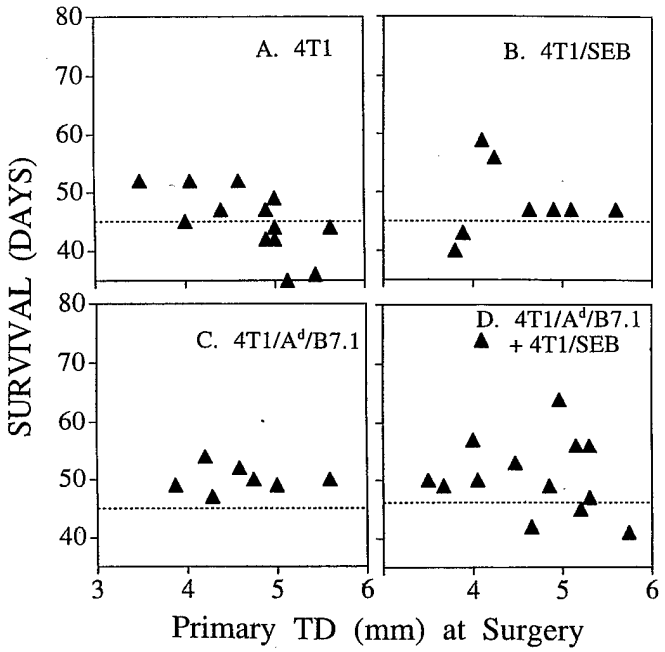

Fig. 3. Immunotherapy of established wild-type spontaneous metastases with a mixture of MHC class II/CD80 and SEB transfectants increases survival. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 live wild-type 4T1 cells. Primary tumors were measured and surgically resected 21 days after parental tumor challenge. Mice were treated every 3–5 days starting at day 28 with i.p. injections of 1×10^6 total cells of irradiated parental 4T1 (13 mice; *A*), 4T1/SEB (8 mice; *B*), $4T1/A^d/B7.1$ (7 mice; *C*), or a 1:1 mixture of $4T1/A^d/B7.1$ plus 4T1/SEB cells (14 mice; *D*). The 1:1 mixture of $4T1/A^d/B7.1$ plus 4T1/SEB therapy group is significantly different from the 4T1 control group ($P = 0.05$, Tukey's Honestly Significant Difference Test).

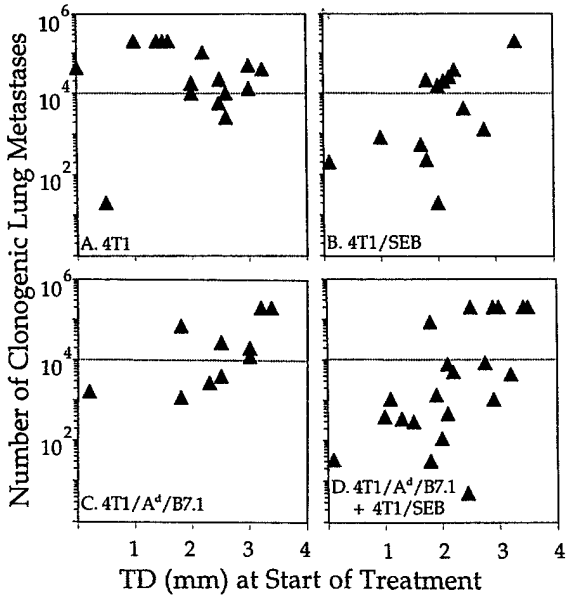

Fig. 4. Immunotherapy of established 4T1 tumors with MHC class II/CD80 and/or SEB transfectants reduces metastatic disease. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 live wild-type 4T1 cells. At 14 days after parental tumor challenge, the TDs were measured and the therapeutic injections started. Mice were treated i.p. twice a week until the time of sacrifice with 1×10^6 total cells/injection of irradiated parental 4T1 (*A*), 4T1/SEB (*B*), 4T1/A^d/B7.1 (*C*), or a 1:1 mix of 4T1/A^d/B7.1 plus 4T1/SEB (*D*) cells. Mice were sacrificed 6 weeks after initial 4T1 tumor challenge, and the number of clonogenic lung metastases was determined. Each *triangle* represents an individual mouse. The 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB therapy group is significantly different from the 4T1 control group ($P = 0.05$, Tukey's Honestly Significant Difference Test).

7×10^3 wild-type 4T1 tumor cells. Starting 2 weeks after challenge, they were given i.p. injections of irradiated vaccine cells (1×10^6 total/injection) twice a week until the day of sacrifice. At the time of sacrifice (6 weeks after the initial primary tumor challenge), primary TD of control-treated mice (*i.e.,* mice given irradiated 4T1 cells) were comparable with TD of transfectant-treated animals (6.5–10.5 mm and 6.2–11.2 mm, respectively; two-tailed $P = 0.61$). Therapy with the transfectants, therefore, does not reduce primary tumor growth, which concurs with our earlier studies using MHC class II or CD80 vaccines alone (3).

To assess the metastatic disease, lungs from the treated mice were removed, dissociated into single cell suspensions, and plated in culture medium containing 6-thioguanine to determine the number of clonogenic tumor cells. Ten days later, the number of clonogenic metastatic cells could be enumerated because 4T1 cells are resistant to 6-thioguanine, whereas normal cells are not resistant and die. As in Fig. 3, we are interested in determining whether primary tumor size effects vaccine efficacy, therefore, the results are plotted as number of clonogenic metastatic cells in the lungs *versus* TD at the start of treatment. A *line* denoting a level of 10,000 tumor cells in the lungs is also included because >85% of untreated tumor-bearing mice contain >10,000 metastatic cells in their lungs after 42 days of primary tumor growth (3). As shown in Fig. 4, administration of the 4T1 transfectants significantly reduces the number of lung metastases (Fig. 4, *B-D*) relative to treatment with wild-type 4T1 cells (Fig. 4*A*). For example, 13 of 16 (81.2%) mice treated with irradiated parental cells (Fig. 4*A*) contained $>10^4$ clonogenic lung metastases, which contrasts 12 of 23 (52.2%) mice treated with either 4T1/SEB or 4T1/A^d/B7.1 (Fig. 4, *B* and *C*) and 6 of 21 (28.6%) mice treated with a mixture of transfectants (Fig. 4*D*). After transforming the number of clonogenic metastases to logarithmic values and analyzing these data using the Tukey's Honestly Significant Difference Test, we found that only treatment with a 1:1 mixture of 4T1/SEB+4T1/A^d/B7.1 cells (Fig. 4*D*) significantly reduced the number of clonogenic lung metastases ($P = 0.05$). Treatment with either 4T1/SEB alone (Fig. 4*B*) or 4T1/A^d/B7.1 alone (Fig. 4*C*) did not significantly decrease the number of clonogenic lung metastases. Previously, we demonstrated that therapy with MHC class II$^+$/CD80$^+$ vaccines statistically significantly reduced clonogenic lung metastases in 50% of mice whose immunotherapy was initiated 9–14 days after tumor challenge but this reduction corresponded to only a 10-fold maximum reduction when compared with the control group (3). In contrast, treatment of mice carrying 14-day established primary and metastatic tumor with the combination therapy of tumor cell transfectants expressing MHC class II, *CD80*, and *SEB* genes decreases spontaneous metastases in the lung by a maximum of 100-fold. Therefore, effective immunotherapeutic treatment of tumor-bearing mice with extensively established spontaneous metastases requires expression of all three molecules.

Reduction of Established Wild-Type Metastases with MHC class II, CD80, and SEB Immunotherapy Requires Both CD4$^+$ and CD8$^+$ T cells. The concept of combining SEB with MHC class II and CD80 was based on the hypothesis that SEB is a potent polyclonal activator of CD4$^+$ T lymphocytes (10, 11) and would provide additional activation signals to CD4$^+$ T cells that have been activated in an antigen-specific fashion by the MHC class II$^+$ CD80$^+$ vaccinating cells. Therefore, we tested the immunotherapy described in Fig. 4 in CD4- or CD8-depleted animals and BALB/c *nu/nu* mice. As shown in Fig. 5, *C* and *D*, depletion of CD4$^+$ or CD8$^+$ T cells (monoclonal antibodies GK1.5 and 2.43, respectively) eliminates the therapeutic effect of the MHC class II$^+$, CD80$^+$, SEB$^+$ vaccine against spontaneous metastases, whereas depletion with control ascites (Fig. 5*B*) has no effect. In addition, the combination vaccine does not reduce metastatic disease in BALB/c *nu/nu* mice (Fig. 5*F*). Collectively, these data demonstrate that the three transfected genes of the cell-based vaccines are working cooperatively to optimally activate both CD4$^+$ and CD8$^+$ T lymphocytes and that these lymphocyte populations are essential for the therapeutic effect.

DISCUSSION

sAgs, including SEB, have been previously recognized as potential reagents for up-regulating T lymphocyte responses against tumors. However, their use has been limited and they have not been combined with other factors that might optimize their therapeutic efficacy. For example, several studies describe redirected T-cell activation using sAgs coupled to tumor-specific monoclonal, anti-idiotypic, or bifunctional antibodies (17–19). SEB has also been administered systemically along with tumor cells, and SEB DNA has been inoculated intratumorally along with cytokine DNA to reduce primary tumor growth (20, 21). In addition, sAgs have been used to activate tumor-draining lymph node T cells *ex vivo* for adoptive transfer into tumor-bearing animals (22, 23). All of these approaches produce some reduction in primary tumor growth and/or decrease in metastatic lesions. However, the test settings have involved relatively small primary tumor and/or very small metastatic tumor burdens, which do not mimic the clinical situation. These results, taken together with the SEB transfected tumor vaccines presented in this study, show that SEB expression alone has only a modest effect on metastatic tumor progression. However, as shown in this study, the antitumor effect of SEB on highly advanced spontaneous metastases is more effective when combined with the cell-based vaccine containing MHC class II and CD80 molecules.

The modified tumor cells may function directly as APCs for the initial activation of tumor-specific CD8$^+$ and CD4$^+$ T cells following immunization. Previous studies demonstrate that both CD8$^+$ and

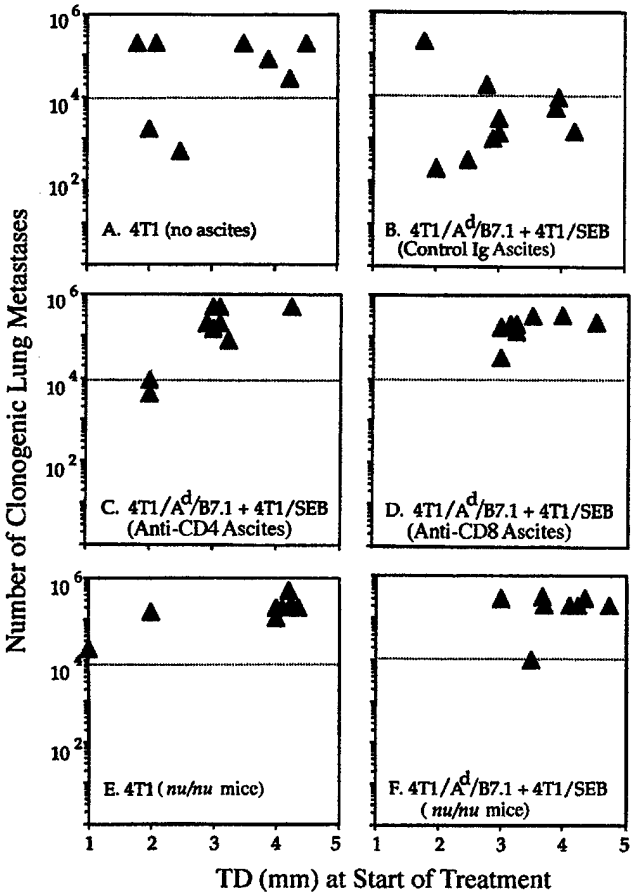

Fig. 5. Reduction of established wild-type metastases with MHC class II, CD80, and SEB immunotherapy requires $CD4^+$ and $CD8^+$ T cells. *A-D*, naïve BALB/c mice received injections s.c. in the abdominal mammary gland of 7×10^3 parental 4T1 cells and, beginning on day 14, were treated as described in Fig. 4 with either 4T1 parental cells (8 mice; *A*) or a 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB (*B–D*). On days 8, 11, and 13 (*i.e.*, before the start of immunotherapy), mice received injections of either control ascites (10 mice; *B*), GK1.5 ascites (9 mice; *C*), or 2.43 ascites (8 mice; *D*). Antibody injections were continued at least once a week for the duration of the experiment. *E* and *F*, BALB/c *nu/nu* mice received injections s.c. in the abdominal mammary gland of 7×10^3 parental 4T1 cells and, beginning on day 14, were treated as described in Fig. 4 with either 4T1 parental cells (8 mice; *E*) or a 1:1 mixture of 4T1/A^d/B7.1 plus 4T1/SEB (8 mice; *F*).

$CD4^+$ T lymphocytes are involved in immunity induced by MHC class II/CD80 vaccines (15) and that MHC class II/CD80 modified tumor cells function directly as APCs for the initial activation of tumor-specific $CD4^+$ T cells (24). Direct presentation of antigen by tumor cells is possible because tumor cell expression of MHC class II molecules in the absence of invariant chain allows for presentation of endogenously synthesized tumor antigens by MHC class II molecules (24, 25). Because the vaccines express MHC class I, class II, CD80, and SEB molecules, antigen-specific and costimulatory signals will be efficiently delivered to $CD8^+$ and $CD4^+$ T cells. Likewise, because the activated $CD8^+$ and $CD4^+$ T cells are in close proximity to each other, there should be an efficient transfer of cytokines between CTLs and T helper cells (see Fig. 6, *right*).

Host-derived APCs are also likely to be involved in $CD8^+$ and $CD4^+$ T lymphocyte activation during vaccine therapy. Because MHC class II serves as a ligand for a sAg (10), it is likely that any host-derived class II^+ cell will bind available SEB. The involvement of host APC is supported by the observation that SEB transfectants alone, which do not express MHC class II, cause a modest therapeutic effect (Fig. 4, *A versus B*). Furthermore, other mouse tumor models have demonstrated that both a class I- and class II-restricted tumor-encoded antigen can be processed and presented indirectly by host-derived APCs (24, 26, 27). Taken together, it is likely that host-derived APCs, capable of migrating to lymph nodes, coordinately present SEB and tumor antigen to both $CD8^+$ and $CD4^+$ T cells (see Fig. 6, *left*).

SEB may also enhance vaccine efficacy because it induces an inflammatory response that stimulates immunity (28). Gene transfer techniques have demonstrated that *in vivo* expression of various sAg (SEA, SEB, and TSST-1) DNAs induces intense inflammatory responses (29). Although systemic administration of sAg (doses >500 μg) typically triggers T-cell release of cytokines such as tumor necrosis factor and lymphotoxin that lead to cachexia (11), we did not see any adverse side effects in SEB-treated mice.

When a sAg, such as SEB, is coexpressed by the MHC class II/CD80 vaccine, additional activation and/or proliferation signals may be delivered to the specifically activated $CD8^+$ and $CD4^+$ T cells. Because SEB binds to the sides of MHC class II molecules and the T cell receptor while antigenic peptide binds within the MHC class II cleft (30, 31), it is feasible that the sAg, tumor antigen-specific, and costimulatory signals are simultaneously received by the T cells. Whereas it is also possible that those signals are not coincident, several studies have shown that activation of T cells by SEB is facilitated or enhanced by B7/CD28 signaling (32–35). Controversy exists over the ability of costimulation to inhibit sAg-induced apoptosis, but one report demonstrates that lipopolysaccharide activation of B cells prevents sAg-induced deletion (36). Regardless of the precise kinetics in which the various activation signals are delivered, coordinate delivery of the three signals improves the efficacy of the vaccines to reduce spontaneous metastatic tumor growth. As a result, T-cell activation may be exceptionally efficient because both direct and indirect antigen presentation occur, thus yielding larger numbers of precisely those $CD8^+$ and $CD4^+$ tumor-specific cells that mediate tumor cell destruction.

New immunotherapies are routinely tested in experimental animal tumor systems. Although such experiments may provide "promising" therapeutic results, tumor regression in animal models does not necessarily predict successful treatment of tumors in human patients. There may be significant physiological and biochemical differences between animals and humans that preclude direct comparison of

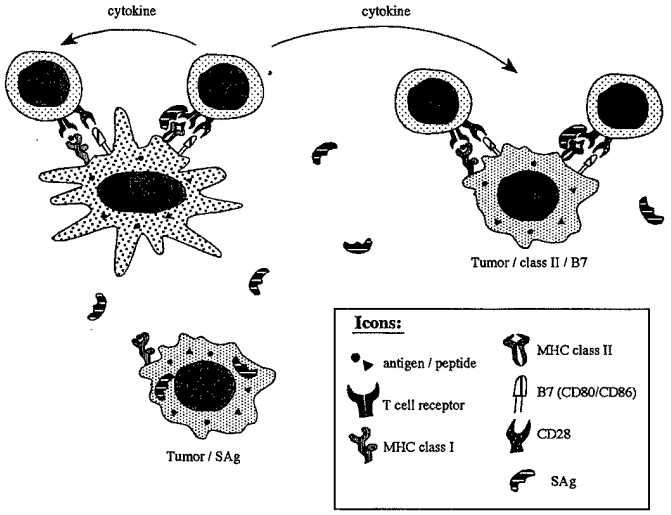

Fig. 6. Proposed mechanism of action by the MHC class II/CD80 and SEB tumor cell-based vaccine. The SEB modified tumor cell (*bottom*) secretes sAgs at the immunization site where the host professional APC (*left*) and the MHC class II/CD80 modified tumor cell (*right*) are able to bind the sAg and activate both $CD4^+$ and $CD8^+$ T cells. As a result, immunization occurs by direct and indirect (cross-priming) antigen presentation.

results, and testing in humans, therefore, will always be required. However, the inability to translate therapies developed in experimental animal models to humans may also be because many of the mouse tumor systems used in immunotherapy studies do not closely model human cancers and, hence, the immunotherapies are not being tested in clinically relevant settings. For example, many immunotherapies are tested in so-called "metastatic" settings, however: (*a*) the extent of metastatic disease is minimal; (*b*) the metastases have not arisen spontaneously; and/or (*c*) the metastases have not been established for significant amounts of time. Furthermore, many commonly used mouse models: (*a*) are not spontaneously metastatic (*e.g.,* CMS-5 fibrosarcoma, RENCA renal cell carcinoma, CT-26 colon adenocarcinoma, SaI sarcoma, and so forth); (*b*) rapidly loose their metastatic potential when cultured *in vitro* (*e.g.,* K1735 melanoma); (*c*) metastasize poorly unless the primary tumor is excised (*e.g.,* B16 melanoma, line 1 carcinoma); or (*d*) rapidly invade the local environment, such that animals die from primary tumor before metastatic disease is established (*e.g.,* B16 melanoma). In contrast, the 4T1 mammary carcinoma is spontaneously metastatic and metastasizes to many of the organs to which human breast cancer metastasizes (*e.g.,* lung, liver, and brain). Also, similar to human mammary carcinoma, 4T1 metastases spread and progress while primary tumor is in place. In addition, following inoculation of a small number of tumor cells (7×10^3) in the mammary gland, lethal metastatic disease develops early (within the first 2–3 weeks) and progresses over several weeks so that immunotherapies can be tested against early or very advanced stage disease. The 4T1 tumor, therefore, is an excellent model for testing experimental immunotherapies. In contrast to our earlier studies with the 4T1 tumor in which relatively early metastases were treated and primary tumor was left in place (3), the studies reported here address very advanced metastatic disease in a postsurgical setting. Although the statistically significant extension of survival time following surgery and administration of immunotherapy was small, we find no comparable studies in the literature in which the efficacy of an immunotherapy is demonstrated in such a clinically relevant model of advanced stage metastatic disease.

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